Signal Transduction Pathways in Hepatocyte Cell Death:
New Targets for Therapy

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Chapter 1

Scope of the Thesis

Cholestatic and steatotic liver diseases comprise a significant share of the total burden of chronic liver diseases. Both cholestatic (e.g., primary biliary cirrhosis: PBC, and primary sclerosing cholangitis: PSC) and steatotic (e.g., non-alcoholic steatohepatitis: NASH) liver diseases are associated with a high morbidity and mortality. Existing therapeutic agents are either not very effective (e.g., steroids or UDCA for PBC) or are associated with severe difficulties (e.g., liver transplantation: donor organ shortage, organ rejection, etc).

One of the reasons for the lack of effective treatment for cholestatic and steatotic liver diseases is the lack of detailed knowledge about the pathogenetic mechanisms of these diseases. Both groups of diseases are characterized by a gradual and progressive loss of viable hepatocytes leading to liver inflammation, fibrogenesis and end-stage liver disease. Cell loss can occur via either apoptosis or necrosis (or intermediate forms). However, extensive controversy exists about these basic knowledge such as the dominant mode of cell death in these disorders. In addition, any intervention or therapeutic strategy should be effective in "diseased" (i.e. steatotic or cholestatic) hepatocytes, since patients with chronic liver diseases usually visit clinicians when their disease is already advanced. The aim of this thesis is to elucidate the mechanisms of cell death in (diseased) hepatocytes, in order to develop strategies to protect (diseased) hepatocytes and prevent liver injury.

In **chapter 2**, the current knowledge on hepatocyte cell death with special emphasis on intracellular organelle-mediated cell death and membrane-bound receptors regulating apoptosis, is reviewed. In **chapter 3**, the dominant mode of cell death in steatotic hepatocytes in an in vivo model of non-alcoholic fatty liver disease (NAFLD) is investigated and it is related to alterations in mitochondrial-mediated signaling pathways.

Angiotensin receptor blockers (ARBs) are suggested as new therapeutics for the treatment of liver fibrosis. In **chapter 4**, the effects of angiotensin II (AT-II) versus ARBs on hepatocytes exposed to bile salts, reactive oxygen species and cytokines are investigated.

Chapter 5 describes the protective effect of pertussis toxin, the Gαi-protein inhibitor, on bile salt- and cytokine-induced apoptotic cell death. This chapter emphasizes the involvement of G-protein coupled receptors (GPCR) in hepatocyte apoptosis and suggests new targets for therapeutic intervention.

Understanding bile salt-induced signaling pathways is crucial for the developmentof novel drugs for cholestatic disorders. **Chapter 6** reports a novel bile salt-induced signaling pathway involving sphingosine kinase-1(SphK1), sphingosine-1 phosphate (S1P, an intracellular lipid molecule potentially acting as a second messenger) and S1P receptors in primary hepatocytes.

Chapter 2

General Introduction

Hepatocyte apoptosis: interplay of intracellular organelles and membrane-bound receptors

Liver diseases belong to the top 10 of leading diseases for humans. In clinical practice, liver injury is divided into acute and chronic diseases, based on the duration or persistence of liver injury. After recovery from acute liver injuries, normal liver function and architecture are restored. In contrast, in most chronic liver injuries liver functions are abnormal and persistent changes in liver architecture occur. Hepatocytes comprise 80% of all liver cells and hepatocyte injury is often central in the pathogenesis of liver diseases [1]. Prolonged hepatocyte injury results in an excessive wound healing response, leading to hepatic inflammation and fibrogenesis. Liver fibrosis is the hall mark of chronic liver diseases that may progress to end-stage liver disease and hepatocellular carcinoma. Toxic bile salts, cytokines, reactive oxygen species and drugs induce hepatocyte injury.

Hepatocyte injury can result in hepatocyte cell death via apoptosis and/or necrosis [2, 3]. Necrosis is a passive process associated with metabolic disruption and energy depletion (loss of ATP), leading to mitochondrial swelling and rupture of the plasma membrane. Subsequently, the cellular content is released into the extracellular environment and systemic circulation, triggering an inflammatory response in the liver. Apoptosis is an ATP-dependent process also known as programmed cell death. Apoptosis is characterized by DNA condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage, resulting in the formation of apoptotic bodies. Apoptotic bodies are cleared by surrounding phagocytosing cells, limiting the inflammatory response [2-5].

Hepatocyte apoptosis is ubiquitous in liver diseases [1, 6-10]. Although apoptosis is primarily a non-inflammatory process responsible for removing excess or damaged cells, apoptosis in pathologic conditions is not controlled and can deteriorate organ function [11]. In the liver, apoptosis contributes to inflammation by promoting Kupffer cell activation. Following uptake of apoptotic bodies, Kupffer cells express death ligands such as TNFα, TRAIL and FasL [12]. All these death ligands may induce apoptosis in hepatocytes via death-receptor induced signaling cascades and thus aggravate liver injury [3, 13, 14]. In addition, activated myofibroblasts (derived from portal fibroblasts and HSCs [15, 16]) are able to engulf apoptotic bodies. Following engulfment of hepatocyte-derived apoptotic bodies, activated myofibroblasts produce profibrogenic cytokines such as TGFβ and type I collagen [17, 18]. Theses data link hepatocyte apoptosis to liver fibrosis in chronic liver diseases, suggesting that the most direct therapeutic strategy for repressing liver fibrosis is to eliminate the cause of hepatocyte injury. Many chronic hepatitis B patients with end-stage liver disease had significant recovery and reversal of liver fibrosis with antiviral therapy and no longer required urgent transplantation (reviewed by [19]). Therefore, removing the cause for hepatocyte injury has become a potential therapeutic strategy for advanced liver diseases. However, effective treatments do not exist for important liver diseases such as primary sclerosing cholangitis, NASH,

ASH and patients with chronic HCV or HBV unresponsive to antiviral therapies. For such patients, anti-apoptotic strategies, which reduce heptocyte injury-mediated inflammation and fibrogenesis, are beneficial [20-22]. Thus, understanding the cellular processes and molecular signaling pathways mediating hepatocyte apoptosis is essential to the development of new therapeutic strategies. In particular, the intracellular organelles and membrane receptors involved in hepatocyte cell death and their interactions are of substantial interest, as a single toxic stimulus often activates several intracellular apoptotic pathways simultaneously. In this review, recent advances in organelle- and membrane receptor-mediated cell death in hepatocytes and potential targets for therapy are discussed.

Organelle-mediated cell death

Mitochondria

Mitochondrial dysfunction is a common observation in several acute and chronic liver diseases such as ASH, NAFLD, drug-induced hepatotoxicity, viral hepatitis, biliary cirrhosis, hepatocellular carcinoma, ischemia/reperfusion injury and transplant rejection [23]. Mitochondria play an essential role in regulating the intrinsic pathway of hepatocyte apoptosis as well as hepatocyte necrosis [3, 24-28]. Mitochondrial permeability transition (MPT) is a key mechanism underlying both apoptosis and necrosis. MPT is characterized by an increase in the permeability of the inner mitochondrial membrane, resulting in the loss of membrane potential, mitochondrial swelling and the rupture of the outer mitochondrial membrane [26]. Opening of the permeability transition pore (mPTP) in the mitochondrial inner membrane is suggested to initiate the MPT. In addition, outer membrane channels such as mitochondrial apoptosis-induced channel (MAC) and the voltage dependent anion channel (VDAC) are directly or indirectly involved in mitochondrial permeabilization during apoptosis and/or necrosis [29]. Mitochondrial outer membrane permeabilization (MOMP) during intrinsic apoptosis leads to the release of apoptotic factors such as cytochrome c, Second Mitochondrial Activator of Caspase/Direct IAP Binding protein with Low pI (SMAC/DIABLO), High-Temperature Requirement protein A2 (HtrA2/Omi), Apoptosis-Inducing Factor (AIF) and endonuclease G [30, 31]. Subsequent activation of effector caspases leads to the proteolysis and typical morphological changes of apoptosis [32]. Although the molecular composition of the mPTP is not completely known, three components are suggested to be directly or indirectly related to the mPTP: the outer membrane channel VDAC [33-35], the adenine nucleotide translocator

(ANT) [36] and cyclophilin-D [37], although studies with knockout animals have raised doubt about the involvement of ANT and VDAC in the mPTP [38, 39]. In addition, recent studies with cyclophilin-D deficient cells strongly suggest that mPTP opening is a consequence, rather than the cause for apoptosis, and validate the original proposal that the mPTP is key in necrotic cell death [37, 40, 41]. Furthermore, the observation that cytochrome c release can occur without the loss of outer membrane integrity indicates that a more selective mechanism of permeabilization such as the formation of a pore in the outer membrane instead of membrane rupture is operating in apoptosis [42-45]. The MAC forms early in apoptosis in the mitochondrial outer membrane and provides a direct pathway for the release of cytochrome c from the inter-membrane space into the cytosol. The MAC is tightly regulated by Bcl-2 family proteins and pro-apoptotic members of this family such as Bak and Bax are components of this channel [31, 42, 43, 43, 45-47]. However, the complete molecular structure of the MAC is not known and it is suggested that the MAC may contain additional components [31, 48]. Whatever their exact composition and function, both the mPTP and the MAC remain potential therapeutic targets to induce cell death in malignancies and prevent cell death in degenerative and ischemia-associated pathologies. Indeed, NIM811 (a non-immunosuppressor cyclosporine-analogue) is reported to decrease liver injury and induce liver regeneration by inhibiting MPT after liver transplantation or massive hepatectomy [49, 50]. NIM811 is also reported to attenuate cholestatic necrosis and apoptosis in BDL rats via inhibition of MPT [51]. Modulation of MPT in a multidrug-resistant hepatocellular carcinoma cell line with the selective MPT opener atractyloside glycoside (ATR) was shown to increase apoptosis in these cells whereas the selective inhibitor of MPT (Cyclosporine A) had the opposite effect [52]. These data suggest that targeting MPT is a potential therapeutic strategy for different liver diseases.

Endoplasmic Reticulum

ER stress is suggested to be an important mechanism in the pathogenesis of chronic liver diseases including NAFLD, ASH, viral hepatitis, drug-induced liver injury, ischemia/reperfusion injury and cholestatic liver disease [53]. The ER is responsible for synthesis, folding, trafficking and maturation of proteins. Under pathologic conditions, the homeostatic equilibrium between the influx of the unfolded proteins and the folding capacity of the ER is disturbed, thereby activating signal transduction pathways between the ER and other intracellular organelles to mediate cellular adaptation to new demands. These series of compensatory responses, termed unfolded protein response (UPR), are conserved in the evolution and promote cellular survival [54]. Glucose

deprivation and the depletion of calcium stores can also induce ER-stress [55-57]. Three membrane sensors in the ER mediate the ER-stress signal transduction: inositol-requiring enzyme-1α (IRE1α), Activating Transcription Factor (ATF) 6 and Protein kinase RNA-like Endoplasmic Reticulum Kinase (PERK). These transmembrane sensors are kept inactivate as long as they are bound to the intraluminal chaperone Glucose-Regulated Protein 78 (GRP78)/BIP [58, 59]. IRE1α is an endoribonuclease that promotes the splicing of X-box binding protein 1 (XBP1) mRNA, resulting in transcription of UPR elements and ER-stress response genes that control ER-associated protein degradation (ERAD) and chaperones [60-62]. PERK induces phosphorylation of eukaryotic translation initiation factor-2α subunit (eIF2α), thereby globally inhibiting protein synthesis [63]. PERK also regulates the transcription of ribosomal RNA via phosphorylation of eIF2 and thereby increases the translation of ATF4. In turn, ATF4 binds to the cAMP-response element (CRE) resulting in the synthesis of C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP) [58, 64, 65]. Active ATF6, following translocation to the Golgi apparatus and RIP (Regulated Intramembrane Proteolysis) translocate to the nucleus and together with sXBP1 and ATF4, activate ER-stress response elements, UPR elements and CRE [66-68]. Upon ER-stress, GRP78 is displaced from the stress sensor to aid in protein folding, leading to the activation of these three ER-stress mediated signaling pathways (i.e. IRE1α, ATF6 and PERK pathways). Prolonged or unchecked ER-stress leads to steatosis, apoptosis and inflammation in the liver [53]. An important feature of the ER-stress response is increased CHOP expression leading to the activation of proapoptotic pathways [69]. Overexpression or microinjection of CHOP protein have been reported to promote cell cycle arrest and/or apoptosis [70, 71]. CHOP can induce the expression of proapoptotic BH3-only protein Bim and the cell surface death receptor TRAIL receptor 2 (also known as death-receptor 5, DR5) and inhibit Bcl-2 transcription [72-74]. CHOP-knockout mice are protected against alcohol-induced hepatocyte apoptosis after alcohol feeding as well as against bile salt-induced hepatocyte apoptosis after bile duct ligation [75, 76]. Acetaminophen (APAP) intoxication has been observed to induce CHOP expression and to cause an intraluminal redox imbalance of the ER, resulting in hepatocyte apoptosis [77]. Importantly, the role of ER stress in APAP-induced necrosis is unknown, although a role in the early activation of JNK and in Ca2+-mediated mitochondrial permeability transition, both key factors in APAP-induced necrosis [78, 79], could be considered.

Several therapeutic interventions, such as chemical chaperones which ameliorate protein folding and antioxidants which counteract oxidative stress, could modulate ER-stress. E.g. the chemical chaperone 4-phenylbutyrate (4PBA) is able to reduce the ER-stress response and decrease

ER-associated caspase 12 activation in livers of mice undergoing ischemia-reperfusion injury [80]. CHOP antagonism is also an obvious target for therapy as CHOP is involved in oxidative stress and apoptosis in hepatocytes. Improved protein folding would be of benefit in disorders of misfolded proteins such as alpha-1-antitrypsin deficiency. Thus agents that ameliorate ER-stress by promoting adaptive UPR signaling or inhibiting ER-stress response-induced apoptosis offer a therapeutic opportunity. Since the ER-stress response has extensive cross-talk with other stress responses, therapeutics aimed at blunting the ER stress response may interrupt this interorganellar cross-talk that operates in many liver diseases [53].

Lysosomes

Lysosomes are involved in necrotic, apoptotic and autophagic cell death. The key factor in determining the type of cell death is the magnitude of lysosomal membrane permeabilization (LMP) and the amount of proteolytic enzymes released into the cytosol [81]. Massive breakdown of lysosomes results in unregulated necrosis, whereas selective permeabilization of lysosomes triggers apoptosis. Several mechanisms for the controlled permeabilization of lysosmes have been proposed. One theory includes the accumulation of lysosomotropic detergents such as sphingosine in the lysosomes, facilitating the release of lysosomal enzymes into the cytoplasm [82]. Another theory involves ROS-mediated lysosomal destabilization. In this theory LMP is suggested to precede mitochondrial dysfunction, thereby creating a feedback loop between mitochondrial-derived ROS and LMP to control cell death. In addition, intralysosomal accumulation of free iron indirectly mediates lysosmal membrane damage via generation of ROS [83, 84]. Translocation of proapoptotic members of the Bcl-2 family such as Bax and Bim to the lysosomes, leading to pore formation and membrane permeabilization (similar to their role in mitochondrial permeabilization) is also proposed as a mechanism for lysosomal leakage [85-88]. LMP associated with cathepsin translocation may directly activate calpains and caspases, triggering classic MOMP- and caspase-dependent apoptosis and/or caspase-independent cell death [89]. Increased lysosomal enzyme activity (e.g., acidic phosphatase) is observed in patients with chronic liver diseases including chronic viral hepatitis, cirrhosis and hepatocellular carcinoma [90]. In addition, LMP and cathepsins have been implicated in cell death in several models of liver injury. For instance, it has been shown that intracellular levels of sphingosine in the liver increases after TNFα treatment, leading to LMP and apoptosis. Interestingly, TNFα or sphingosine could not induce LMP in hepatocytes from cathepsin-B knockout livers, suggesting that cathepsins may also be inducers of LMP. Cathepsins

may directly induce LMP acting from the inside and/or outside of the lysosomes or they may participate in an amplification loop in which LMP induces cathepsin activation, and cathepsin then triggers further LMP [88, 91]. Free fatty acids and bile salts can also induce LMP-dependent cell death [92-95]. Glycochenodexycholic acid (a cholestatic bile salt) induces LMP, cathepsin-B translocation, caspase activation and cell death in hepatocytes in animal models of cholestasis [92, 96]. Excessive accumulation of saturated free fatty acids in the liver has been reported to directly induce mitochondrial dysfunction and oxidative stress via LMP and activation of cathepsin B [95]. Translocation of non-heme iron from lysosomes to the mitochondria is reported to play an important role in oxidative-stress induced hepatocellular damage, identifying this pathway as a potential therapeutic target to combat oxidative stress-mediated hepatotoxicity [97]. Cymotrypsin B, a lysosomal enzyme originally described in pancreas, was also found in rat liver lysosomes. Interestingly, cymotripsin B was reported to cleave the non-apoptotic Bid into pro-apoptotic truncated Bid (tBid) at neutral pH. tBid then translocates to mitochondria, leading to MOMP and cytochrome c release. Knockdown of cymotripsin B or pretreatment with the cymotripsin B inhibitor, N-p-tosyl-L-phenylalanine chloromethyl ketone, reduced TNFα-induced apoptosis in hepatocytes [98]. As noted before for ER-stress, it is important to appreciate that disturbances in redox status, steatosis, oxidative stress, inflammation and mitochondrial injury all affect LMP. Interorganellar signaling pathways, death-receptor mediated signaling and lysosomal proteases such as cathepsin B can all be activated by LMP. Therefore, therapeutics aimed at reducing LMP may interrupt all these signaling pathways, thereby reducing liver inflammation and hepatocyte damage.

Receptor-mediated cell death

Membrane death receptors

Death receptors belong to the TNF/Nerve Growth Factor superfamily and are involved in death ligand-mediated cell death. TNFα, Fas ligand (FasL) and Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) are death ligands that signal via binding to their membrane-bound receptors including: Fas, Tumor Necrosis Factor Receptor (TNFR1 and TNFR2), and TRAIL receptors (R1/DR4 and R2/DR5). Receptor-ligand binding triggers receptor trimerization at the cell surface, providing a platform at the cytoplasmic side of the plasma membrane (termed Death Domains) which recruits adaptor proteins such as Fas-associated death domain (FADD). The interaction between the death domains (DD) of death receptors and adaptor proteins leads

to the activation of caspase-8 and subsequent cleavage of Bid to tBid. Translocation of tBid to mitochondria results in mitochondrial permeabilization and activation of the apoptotic cascades. In addition, TNFα-induced Bid-dependent and TRAIL-induced Bax-dependent LMP has been described. LMP then leads to cell death [88, 99]. Although death receptors do not induce ER stress, ER stress-mediated regulation of TRAIL receptor (DR5) expression has been reported [73, 100]. Therefore, interaction between death receptor signaling and intracellular organelles plays an important role in cell death.

Several liver cells including hepatocytes express Fas (CD95/ Apo-1) [101-103]. Fas is activated upon binding of membrane-bound FasL or soluble FasL. FasL is expressed on several immune cells including cytotoxic T lymphocytes and natural killer (NK) cells [104]. FasL then activates Fas-mediated cell death, leading to the removal of unwanted hepatocytes such as virus-infected hepatocytes and cancer cells by immune cells [105]. However, excessive Fas-induced cell death leads to liver failure. Indeed, injection of Fas agonistic antibody to mice induces fulminant hepatic failure. In addition, it was shown that this toxicity is regulated by anti-apoptotic and pro-apoptotic Bcl-2 members [106-108]. Elevation of soluble FasL occurs in patients with acute liver failure (such as drug-induced liver injury and acetaminophen-induced liver injury) and FasL and/or Fas receptor expression is increased in many chronic liver diseases including chronic viral hepatitis and alcoholic liver diseases [109-112]. In summary, Fas/FasL induced apoptotic cell death plays a crucial role in liver pathogenesis.

TNFR1 and TNFR2 are both expressed on hepatocytes, but only TNFR1 expresses a DD [113]. Importantly, TNFR1 activation in hepatocytes can trigger both apoptotic and survival signaling. TNFα-exposure leads to the rapid formation of a DISC (Death Inducing Signaling Complex) composed of TNFα, the TNFR-associated death domain adaptor molecule (TRADD), the Fas-associated death domain adaptor molecule (FADD), caspase-8, TNFR-associated factor -2 (TRAF2) and receptor-interacting protein (RIP). Interestingly, it was shown that TNFR1 and some DISC components also appear inside mitochondria within 30 minutes after TNFα-exposure, suggesting that TNFα-mediated signaling includes the translocation of TNFR1 (and associayed proteins) to mitochondria [114]. In contrast, TRADD suppresses apoptosis by recruiting RIP, TRAF2 and TRAF5. Immediate binding of RIP and TRAF2 to TRADD lead to the activation of Nuclear Factor-κB (NF-κB) and transcriptional activation of prosurvival genes including Bcl-xL, A1, XIAP and cFLIP [115, 116]. TNFα/TNFR2 signaling is involved in the activation of hepatocyte DNA synthesis and proliferation as well as in the regulation of FasL-dependent clearance of virus infected hepatocytes by cytotoxic T lymphocytes (CTL) [117, 118]. Interestingly, mice lacking both TNFR1

and TNFR2 are resistant to anti-Fas induced-fulminant hepatic failure [119]. These data suggest that TNFα/TNFRs interactions are critical for the proper functioning of CTL activity in the liver and clearance of infected and/or damaged hepatocytes.

TRAIL is emerging as a key mediator of hepatic injury during inflammatory disorders of the liver [120]. Early studies demonstrated that different cells have different sensitivity to TRAIL-induced cell death. Whereas cancer cells are sensitive to TRAIL-induced toxicity, normal (non-transformed) cells were resistant to its apoptosis-inducing effects. Hence, TRAIL-induced selective cancer cell death without damage to the adjacent normal tissue was suggested to be an efficient anti-cancer therapy [121, 122]. Indeed, TRAIL, DR4 and DR5 (TRAIL receptors) messenger RNA are expressed at low levels in normal human liver and the expression of the receptors at the protein level is difficult to detect. However, emerging data indicate that DR4 and DR5 expression is upregulated in several liver diseases such as steatosis and HCV- and HBV-infection [123-126]. HIV and/or the ligation of HIV glycoprotein gp120 to CXCR4 on hepatocytes also selectively increases DR5 expression and suggest that HIV infection renders hepatocytes more susceptible to apoptotic cell death in liver diseases associated with enhanced TRAIL expression such as HBV, HCV or steatohepatitis [127]. Increased DR5 expression in experimental models of NASH is associated with the activation of p53 and ER-stress induced CHOP expression by free fatty acids such as palmitate [128, 129]. In addition, bile acids can increase DR5 expression and inhibit cFLIP (inhibitor of DR5-induced signaling) function, thereby sensitize hepatocytes to TRAIL mediated cell death in cholestatic livers [130, 131]. Thus, the safety of TRAIL administration to humans with underlying liver disease is questionable. The development of selective DR4 agonistic antibodies to treat cancer in patients with underlying liver diseases could be an attractive strategy, considering the possibility of DR5-dependent TRAIL toxicity in liver diseases.

In summary, excessive generation of inflammatory mediators and activation of death-receptor mediated apoptotic pathways can exacerbate underlying liver diseases. Therefore, therapies targeting death receptor-mediated hepatotoxicity in combination with efforts to remove the cause of the disease (e.g., virus, toxic bile acids, free fatty acids and drugs) may be a beneficial strategy in the treatment of liver diseases.

Epidermal growth factor receptor

The epidermal growth factor receptors (EGFR; ErbB-1; HER1 in humans) are the cell-surface receptors for members of the epidermal growth factor family (EGF-family) of extracellular protein

ligands. The EGFR is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2),Her 3 (ErbB-3) and Her 4 (ErbB-4) [132]. Growth factor receptors such as the EGFR are involved in both cell proliferation and cell death in the liver [133]. EGF functions as an endocrine factor. It is produced by a variety of cells and EGF/EGFR interaction is essential for liver regeneration [134]. Ligand-dependent EGFR activation also plays a role in hepatic tumorigenesis and EGFR-targeting agents such as monoclonal antibodies (e.g., cetuximab and panitumumab) and tyrosine kinase inhibitors (e.g., gefitinib and erlotinib) are suggested in the treatment of liver carcinomas [135, 136]. In addition to ligand-dependent EGFR activation, ligand-independent EGFR activation in the liver has been described. For instance, hydrophobic bile acids induce reactive oxygen species (ROS)-dependent EGFR tyrosine phosphorylation in hepatocytes, leading to the activation of downstream mitogen-activated protein kinases (MAPK) [14]. Hydrophobic bile acids also induce extracellular kinase regulated (ERK)-dependent hepatic stellate cell (HSC) proliferation via ROS-dependent EGFR activation [137]. As described above, ligand-independent EGFR activation plays an important role in liver pathogenesis. In hepatocytes, ligand-independent EGFR activation also contributes to apoptosis [133]. Several mechanisms for ligand-independent EGFR activation have been described such as EGFR transactivation by Fas ligand (FasL)[138, 139], hydrophibic bile salts (deoxycholate, glychocenodeoxycholate, etc) or hyperosmolarity [14, 138, 139]. Ligand-independent activation of EGFR is followed by JNK-dependent EGFR/Fas association, a process that requires ROS-dependent Yes activation, followed by Yes-mediated EGFR transactivation [138-142]. EGFR/Fas association results in EGFR-mediated tyrosine phosphorylation of Fas (death receptor), leading to Fas oligomerization, membrane translocation, DISC formation and execution of apoptosis [138, 139, 143].

These data suggest that agents that inhibit ligand-independent activation of EGFR such as tyrosine kinase inhibitors may have substantial anti-apoptotic effects in hepatocytes. Interestingly, recent evidence suggests that the EGFR can also function as a host cofactor for HCV entry and tyrosine kinase inhibitors show substantial antiviral activity [144]. Chronic viral hepatitis is associated with activation of inflammatory mediators and accumulation of immune cells in the liver which can induce hepatocyte apoptosis/necrosis in non-infected hepatocytes via activation of death-receptor signaling pathways. Thus inhibition of receptor tyrosine kinase (such as EGFR) may constitute an attractive novel approach in the treatment of chronic liver diseases, in particular chronic viral hepatitis due to their antiviral activity and anti-apoptotic effects in healthy hepatocytes.

G-protein coupled receptors

GPCRs are the largest family of membrane proteins. More than 300 GPCRs have been reported in humans and rodents [145]. GPCRs transduce extracellular signals to intracellular effector pathways. Upon activation by agonists, GPCRs activate heterotrimeric G-proteins (Gαβγ). These subunits subsequently activate second messengers (e.g. cAMP, Ca^{2+} and protein kinases), relaying the GPCR induced-signal to the intracellular targets. Heterotrimeric G-proteins are divided into 4 families (i.e., Gαs, Gαi, Gαq/11 and Gα12/13) based on the Gα subunit sequence and signaling activity [146]. Many GPCRs such as lysophosphatidic acid (LPA), sphingosine-1 phosphate (S1P) and orexin (OXR) receptors are involved in the regulation of apoptosis in cancer cells [147-149]. Signaling components acting downstream of the GPCRs or G-proteins (e.g., arrestin and adaptor protein 2) may mediate anti-apoptotic events following stimulation of GPCRs [150, 151]. Activation of GPCRs regulates apoptosis in cancer cells via interaction with different intracellular regulators of apoptosis such as MAPKs, NF–κB and p53- associated pathways [152]. E.g., LPA-dependent signaling decreases the nuclear localization and cellular abundance of p53, leading to resistance of lung carcinoma cells to apoptosis [153]. Stimulation of endogenous muscarinic receptors in SH-SY5Y cells (human neuroblastoma cell line) was able to inhibit p53 translocation to the mitochondria and p53 phosphorylation at serine 15 and 37, thereby protecting these cells against DNA-damaging agents [154]. Activation of the receptors for LPA, ET1 (endothelin) and angiotensin II can activate NF-κB-regulated pathways. NF-κB then mediates either anti-apoptotic or pro-apoptotic responses, depending on the stimulus and the cell type [115, 155-158]. In the liver, activation of GPCRs also mediates both apoptotic and anti-apoptotic responses. E.g., S1P receptors mediate both apoptotic and anti-apoptotic pathways in human hepatic myofibroblasts [159]. Free fatty acids such as palmitic acid mediate apoptosis in hepatocytes via phospholipase A2 (PLA2)/lysophosphatidylcholine (LPC)/LPA-dependent signaling [160]. However, the exact signaling pathways downstream of GPCRs that regulate apoptosis in hepatocytes are yet unknown. Nevertheless, excellent therapeutic benefits have been observed with some GPCR-based drugs in cancer therapy: the endothelin A receptor antagonists -ZD4054 and atrasentan- show potent antitumor efficacy for ovarian and prostate cancer [152, 161-163]. GPCR-based drugs may also show therapeutic benefits in the regulation of apoptosis in chronic liver diseases and liver tumors.

Conclusions

Understanding the cellular mechanisms that control death in hepatocytes is of clinical and

scientific importance in the development of novel therapies. Hepatocyte cell death appears to be at the cross-roads of several cellular mechanisms rather than the end-point of a linear cascade of events. One particular toxic stimulus may simultaneously promote the activation of several types of membrane-bound receptors as well as organelle-mediated signaling pathways, leading to apoptosis. In addition, several toxic stimuli often play a role in the pathogenesis of chronic liver diseases (e.g., free fatty acids and inflammatory mediators in NASH). Thus, anti-apoptotic therapeutics aimed at one particular pathway may be more effective when used in combination with other interventions.

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Chapter 3

Mitochondrial permeability transition pore proteins are up-regulated in fatty hepatocytes that show an increased susceptibility to necrosis

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Abstract

Background & Aim: Excessive hepatocyte death in non-alcoholic fatty liver disease (NAFLD) triggers inflammatory responses in the liver. Mitochondrial permeability transition (mPT) is the crucial event in the regulation of cell death and is initiated by mPT pore (mPTP) opening. Up-regulation of mPTP proteins results in increased susceptibility to cell death, in particular necrosis. Whether expression of mPTP proteins is changed in fatty hepatocytes is not known. Here, we analyzed the expression pattern of mPTP proteins in fatty hepatocytes and we studied their susceptibility to apoptotic and necrotic cell death.

Methods: Expression of the mPTP proteins VDACs (Vdac1-3), ADP/ATP translocase (Ant-1) and Cyclophilin D (Ppid) was analyzed in hepatocytes isolated from leptin receptor-deficient Zucker rats, an established model of NAFLD, with lean Zucker rats serving as controls. Primary hepatocytes were exposed to glycochenodeoxycholic acid (GCDCA) and tumor necrosis factor-α (TNFα)/actinomycin D (ActD) to induce apoptosis. Apoptosis (caspase-3 activity, acridine orange staining) and necrosis (LDH leakage, Sytox green staining) were quantified.

Results: mRNA and protein expression of Ant-1 and Ppid were significantly increased in fatty hepatocytes. Compared to lean hepatocytes, fatty hepatocytes showed an increased level of necrosis during standard culture conditions ($P<0.05$), even though their viability directly after isolation was the same. In contrast, fatty hepatocytes showed a reduced sensitivity towards GCDCA-induced apoptosis, while their susceptibility to TNFα/ActD-induced apoptosis was unchanged.

Conclusion: Cyclophilin D and Ant-1, two crucial components of mPTP, are up-regulated in fatty hepatocytes, which coincides with a clear increase in the susceptibility to necrosis.

Key words: non-alcoholic fatty liver disease, necrosis, mitochondrial permeability transition, inflammation, cell death.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common feature of the metabolic syndrome. The prevalence of NAFLD is increasing worldwide and is estimated to affect approximately 20% of the population in developed countries [1]. Histopathological features of NAFLD range from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is characterized by the presence of inflammation in addition to steatosis and may progress to cirrhosis, hepatocellular carcinoma, and eventually death [2]. Fatty livers are vulnerable to injury from various causes and the liver-related morbidity and mortality increases substantially once hepatitis develops [3, 4]. Therefore, the transition from steatosis to steatohepatitis is an important event in the progression of chronic fatty liver disease. By inducing hepatocyte injury, lipotoxicity, oxidative stress, pro-inflammatory cytokines, bacterial lipopolysaccharides and cholestasis may all trigger the progression of steatosis to steatohepatitis [1].

Hepatocyte injury is one of the important features observed in NASH [2]. Hepatocyte death can happen via apoptosis, necrosis or a combination of both modes of cell death. Apoptosis results in the fragmentation of the cell into apoptotic bodies, which are cleared by surrounding phagocytes thereby minimizing inflammation during liver injury [5]. Necrosis leads to mitochondrial and cellular swelling, the rupture of the plasma membrane and the subsequent release of the cellular content into the extracellular environment, resulting in an inflammatory response in the liver [5]. Mitochondria play a crucial role in both types of cell death [6]. Onset of the mitochondrial permeability transition (mPT) is suggested to be the common mechanism for both apoptosis and necrosis. Opening of permeability transition pores (mPTP) in the mitochondrial membrane is one of the initiating events in the mPT [7-9]. Pore opening leads to a non-selective diffusion of proteins across the mitochondrial membrane and subsequently cell death [8-10]. Importantly, it is suggested that mPTP plays a crucial role in necrosis rather than apoptosis [11, 12] and cells overexpressing the mPTP-associated protein, Cyclophilin D, are more susceptible to necrotic cell death [13]. In addition, mice overexpressing Cyclophilin D show mitochondrial swelling and spontaneous cell death in vivo [14]. Mitochondrial dysfunction and alterations in mPT have been suggested as predisposing mechanisms in the transition from steatosis to steatohepatitis (likely via modulating hepatocyte cell death in fatty livers) [15-18]. In the present study, we demonstrate that mPTP proteins, Cyclophilin D and Ant-1, are up-regulated in fatty hepatocytes from Zucker rats resulting in a predisposition of these cells to necrosis even under control conditions in the presence of normal

cellular and extracellular ATP levels. We further demonstrate that fatty hepatocytes show a reduced sensitivity to bile acid-induced apoptosis, but they do not have an altered sensitivity to cytokine-induced apoptosis.

Materials and Methods

Animals

Specified pathogen-free male Zucker rats (fa/fa) and their corresponding lean (FA/FA or FA/fa) male littermates (8-10 weeks of age) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Animals were caged in animal rooms with alternating 12-hour light/dark period, free access to standard laboratory chow and water for 2 weeks. Fatty Zucker rats and lean Zucker rats were weighed prior to the hepatocyte isolation. All experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

Rat hepatocyte isolation

Hepatocytes were isolated from Zucker fatty rats and Zucker lean controls and cultured in multiwell plates (Greiner bio-one) in William's E medium in a humidified incubator at 37 ^{o}C and 5% CO_2 as described before [19]. Aliquots of approximately 1 million cells were snap-frozen in liquid nitrogen immediately after isolation (t_0 hepatocytes) for RNA and protein analysis.

Experimental design

Experiments were started after the attachment period of 4 hours. Monolayers of cultured primary hepatocytes were treated with 50-200 μmol/L GCDCA (Sigma-Aldrich) for 2- 4 hours. Alternatively, hepatocytes were treated for 16 hours with 20 ng/ml recombinant murine tumor necrosis factor α (mTNFα, R&D Systems, Abingdon, UK) in combination with 200 ng/ml of the transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, the Netherlands). Actinomycin D was added to cultured hepatocytes 30 minutes prior to the apoptotic stimulus. Every experimental condition was performed in triplicate and each experiment was repeated at least

three times using hepatocytes from different rats. Cells were harvested at the indicated time points as described previously [20]. Hepatocytes were isolated from different fatty Zucker rats (n = 9) and lean Zucker rats (n = 8).

Hepatocyte triglyceride measurement

Cellular triglyceride content was determined in t0 hepatocytes with a commercially available kit (Wako Chemicals, Neuss, Germany) after lipid extraction as described before [21].

ATP measurement

Rat hepatocytes were cultured in 96-well plates. Cells were treated according to the experimental design after the attachment period of 4 hours. ATP levels were measured using the Cell Viability Kit (CellTiter-GIo® Luminescent Assay, Promega, Madison, USA) according to the manufacturers instruction.

Apoptosis and necrosis assays

Caspase-3 activity was measured as described previously [20]. The arbitrary fluorescence unit (AFU) was corrected for the amount of protein. Protein concentration was determined using the Bio-Rad protein assay kit. LDH release from hepatocytes was measured in the supernatant of medium of cultured cells at different time points after hepatocyte isolation. LDH activity was determined spectrophotometrically at 340 nm. Sytox green (Invitrogen) and acridine orange (Sigma-Aldrich) were used to visualize necrotic and apoptotic cell death, respectively, as described before [19].

Quantitative PCR

RNA isolation, reverse transcription PCR and quantitative PCR (qPCR) were performed as described previously [19]. Each sample was analyzed in duplicate. 18S mRNA levels were used as endogenous control. Primers and probes are listed in Table 1.

Table 1: list of probes and primers used for Q-PCR analysis

18 S Rat	Sense Antisense Probe	5'-CGGCTACCACATCCAAGGA- 3' 5'-CCAATTACAGGGCCTCGAAA-3' 5'-FAM-CGCGCAAATTACCCACTCCCGA- TAMRA3'
Ant-1 Rat	Sense Antisense Probe	5'-TTGACACTGTCCGTCGTAGGAT-3' 5'-AACTGTCCCCGTGTACATAATATCAG-3' 5'-FAM-CCGGCCAGACTGCATCA-TAMRA3'
Ant-2 Rat	Sense Antisense Probe	5'-TACTTCGGTATCTATGACACTGCAAA-3' 5'-CCAGCTGATGAAGATGTGAGTATTCT-3' 5'-FAM-ATGCTCCCGGATCC- TAMRA3'
Vdac-1 Rat	Sense Antisense Probe	5'-GGGTACAAGAGGGAGCATATCAA-3' 5'-TGAGGGCCCAGTGATGTCA-3' 5'-FAM-TCCACATCACAGCCCA-TAMRA3'
Vdac-2 Rat	Sense Antisense Probe	5'-GGAGTGGGCTATACTCAGACTCTGA-3' 5'-AGCATTAAAGCTCTTCCCGTCTAC-3' 5'-FAM- CTGGTGTGAAGCTTAC-TAMRA3'
Vdac-3 Rat	Sense Antisense Probe	5'-AGGCTGCCAAGGATGTCTTTAA-3' 5'-CCACTACAAGACTTGGTTTTCAGATC-3' 5'-FAM-ATTTTGACCATGCCAAACCCATACCCTT-TAMRA3'
Ppid Rat		Assay on Demand: Rn01458749 (Invitrogen)

Western-blot analysis

Western blot analysis was performed as described previously [19]. Expression of selected proteins was assessed using polyclonal rabbit antibodies against Cyclophilin D (Ppid; Thermo Scientific), Ant-1 (Thermo Scientifc) or monoclonal rabbit antibody against total Vdac (Cell Signaling) at a dilution of 1:1000. Horse radish-peroxidase conjugated goat anti-rabbit Ig (DAKO, Denmark) was used as a secondary antibody at a dilution of 1:2000.

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± SD. Student t-test

or ANOVA were used to determine the significance of differences between experimental groups. A P-value of less than 0.05 (P<0.05) was considered statistically significant.

Results

Zucker fatty rats have a higher hepatic triglyceride content compared to lean rats

The Zucker fatty (fa/fa) rat is genetically homozygous for a mutation in the leptin receptor gene and hence develops severe obesity, hyperinsulinemia and fatty liver disease [22]. NAFLD is characterized by the accumulation of triglycerides (TG) within hepatocytes. In accordance, obese (fa/fa) rats at the age of 10-12 weeks were significantly heavier than lean (FA/fa; FA/FA) control rats prior to the hepatocyte isolation (335 ± 82.66 vs. 255 ± 61.84 gr, respectively, P < 0.05, Figure1A). We then measured the triglyceride (TG) content in hepatocytes directly after the isolation (t0 hepatocytes). Fatty hepatocytes had almost 4-fold higher amounts of TG compared to hepatocytes from lean controls (3.917 ± 1.826 vs. 1.061 ± 0.7149 mmol TG/ mg protein, respectively, P< 0.05; Figure1B).

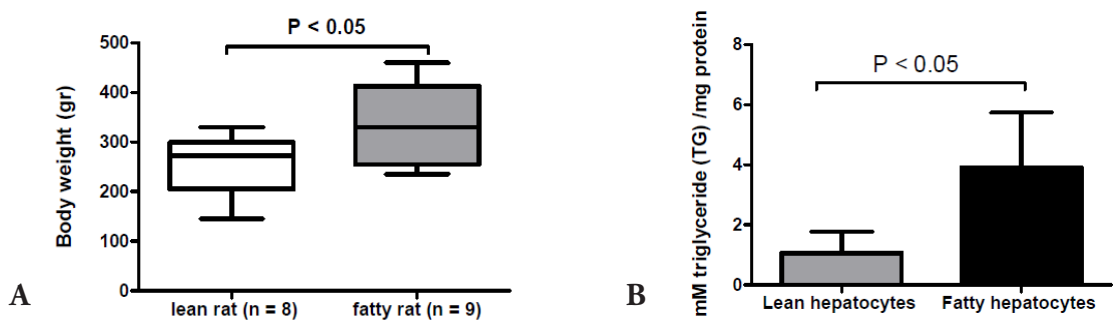

Figure1: Zucker fatty rats are heavier than Zucker lean rats and hepatocyte triglyceride (TG) content is increased. **(A)** Body weight of Zucker fatty rats (n = 9) and Zucker lean rats (n = 8) was measured at the age of 10-12 weeks prior to the hepatocyte isolation. **(B)** The TG content was determined in hepatocytes from fatty and lean rats directly after isolation (t_0 hepatocytes). The TG content (mM) was corrected for the amount of protein (mg) and expressed as mM/ mg.

Mitochondrial permeability transition pore (mPTP) proteins are higher expressed in fatty hepatocytes compared to lean hepatocytes predisposing them to necrosis

The mPTP is known to be a regulator of mitochondrial integrity during cell death. Recent studies have linked the mPTP to events of late apoptosis and necrosis [7, 9]. Overexpression of the

mPTP protein Cyclophilin D (Ppid) increases the susceptibility of cells to necrosis [13, 23]. We compared the expression of the mPTP-associated proteins Cyclophilin D (Ppid), voltage-dependent anion channel (Vdac: isoforms 1-3) and adenine nucleotide translocator (Ant: isoforms 1 and 2) in t_0 fatty hepatocytes and t_0 lean hepatocytes. Interestingly, fatty hepatocytes expressed significantly higher mRNA levels of Ppid, Vdac-1, Vdac-3 and Ant-1 compared to lean hepatocytes (P<0.05; Figure2 A).

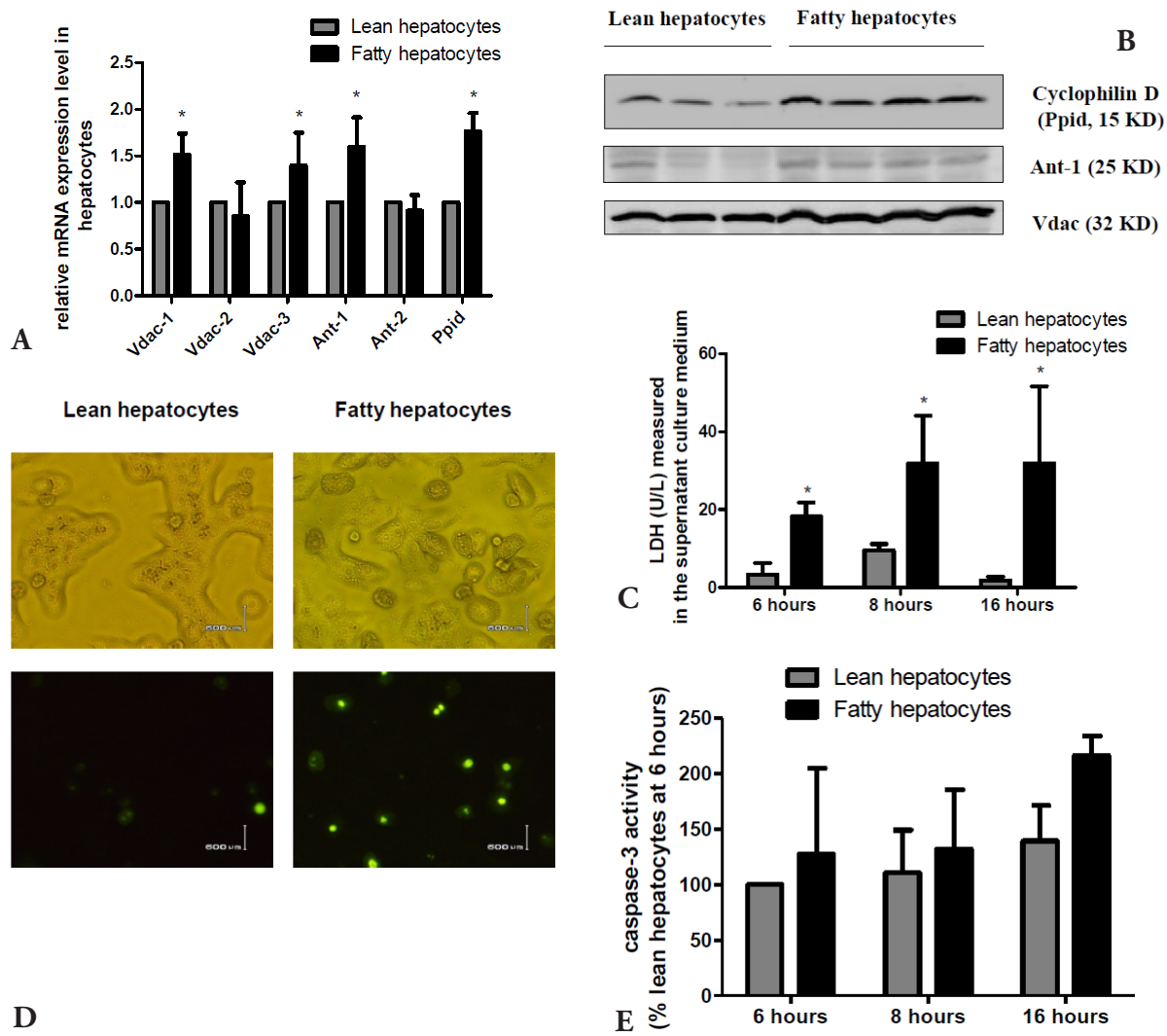

Figure2: mPTP related proteins are up-regulated in fatty hepatocytes and fatty hepatocytes are more susceptible to necrosis than lean hepatocytes. (A) Expression pattern of mPTP proteins in fatty and lean hepatocytes. Q-PCR analysis for Vdac-1, Vdac-2, Vdac-3, Ant-1, Ant-2 and Cyclophilin D (Ppid) using total RNA isolated from fatty and lean hepatocytes immediately after isolation (t0 hepatocytes). * P < 0.05 for fatty hepatocytes vs lean hepatocytes. **(B)** Western-blot analysis for Cyclophilin D (Ppid), Ant-1 and total Vdac in cell lysates of t_0 hepatocytes isolated from fatty and lean rats. Equal amounts of protein were loaded. **(C)** LDH leakage was determined in the supernatant of cultured fatty and lean hepatocytes at the indicated time points. * P<0.05 fatty hepatocytes vs lean hepatocytes. **(D)** Necrotic hepatocytes visualized by Sytox green staining at 16 h after the hepatocyte isolation. Lean hepatocytes (left panels) and fatty hepatocytes (right panels). **(E)** Caspase-3 activity in cultured fatty and lean hepatocytes at indicated time points. Caspase-3 activity is expressed as percentage of the caspase-3 activity in lean hepatocytes at 6 h.

The fatty hepatocytes also expressed higher protein levels of Cyclophilin D (Ppid) and Ant-1 compared to the lean hepatocytes, whereas there was no difference in the protein expression of total Vdac in both types of hepatocytes (Figure2 B). These data indicate that Cyclophilin D and Ant-1, two crucial components of mPTP [9], are up-regulated in fatty hepatocytes even under baseline conditions which may predispose fatty hepatocytes to necrosis.

LDH leakage from hepatocytes is an indicator of cellular necrosis [24, 25]. We measured LDH leakage in the supernatant of cultured hepatocytes at 6 h, 8 h and 16 h after the hepatocyte isolation. The initial viability for lean and fatty hepatocytes was the same (80 %). However, fatty hepatocytes released significantly more LDH into the medium compared to lean hepatocytes at all time points tested ($P<0.05$; Figure2 C), indicating that fatty hepatocytes are predisposed to necrosis even under normal conditions. In accordance, Sytox green staining showed that the number of necrotic fatty hepatocytes at 16 h after isolation was increased compared to lean hepatocytes (Figure2 D). Importantly, caspase-3 activity in both fatty and lean hepatocytes was equally low at these time points (Figure2 E), indicating that the isolated hepatocytes were not apoptotic.

Fatty hepatocytes show reduced susceptibility to GCDCA-induced apoptosis compared to lean hepatocytes, despite similar total ATP levels

GCDCA (50 μM) induces caspase-3 activity in fatty and lean hepatocytes peaking at 2 h (data not shown). We compared the effect of different concentrations of GCDCA (50-200 μM) in fatty and lean hepatocytes at this time point. The GCDCA-induced caspase-3 activity in fatty hepatocytes was significantly lower (50%, $P<0.05$) compared to lean hepatocytes at 50 μM and 100 μM GCDCA (Figure3 A), indicating that fatty hepatocytes show decreased GCDCA-induced apoptosis compared to lean hepatocytes. At higher GCDCA concentration (200 μM) the primary mode of cell death shifts to necrosis [19] and thus less caspase-3 activity is detected in both lean and fatty hepatocytes (Figure3A). To establish true apoptosis, GCDCA-treated hepatocytes were stained with acridine orange after 2 hours exposure to 50-200 μM GCDCA. Cellular blebbing, nuclear fragmentation and condensation, all markers for end-stage apoptosis, were readily detectable at the concentration of 50 μM GCDCA in lean hepatocytes and increased upon exposure to higher concentrations of GCDCA (Figure3B). In contrast, these features of apoptosis were not present in fatty hepatocytes upon exposure to 50 μM GCDCA and were less prominent in fatty hepatocytes upon exposure to higher GCDCA concentrations compared to lean hepatocytes (Figure3B)

confirming that fatty hepatocytes show reduced sensitivity to GCDCA-induced apoptosis.

It has been suggested that lower hepatic ATP concentrations results in necrotic cell death of hepatocytes [26].

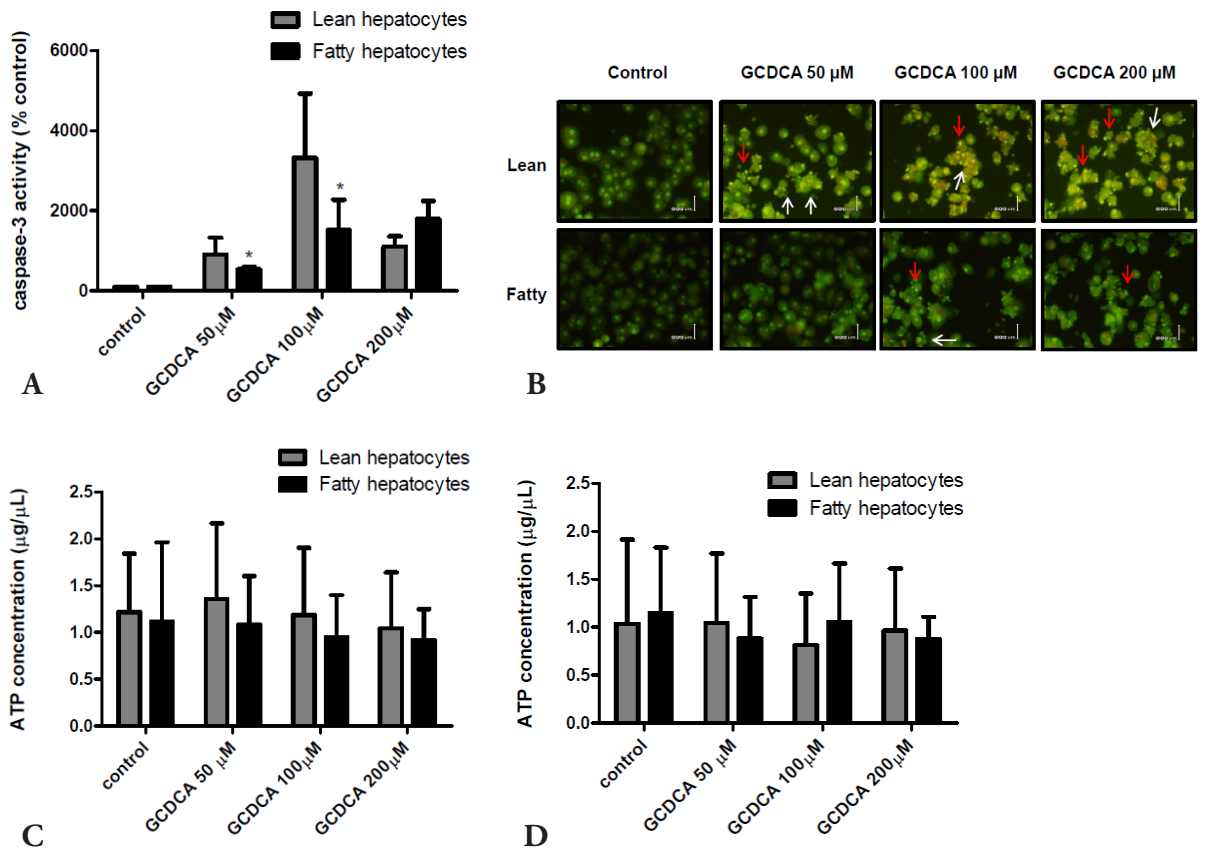

Figure3: Fatty hepatocytes show reduced susceptibility to glycochenodeoxycholic acid (GCDCA)-induced apoptosis, which is not due to lower basal ATP levels or an altered decline pattern of ATP following GCDCA exposure. (A) Caspase-3 activity in primary rat hepatocytes. Fatty heptocytes and lean hepatocytes were exposed to 50-200 µ of GCDCA for 2 hours. * $P < 0.05$ for fatty hepatocytes vs lean hepatocytes. **(B)** Nuclear morphology and cellular blebbing of hepatocytes visualized by acridine orange staining. Nuclear fragmentation, condensation (red arrows) and cellular blebbing (white arrows) are less abundant in fatty hepatocytes (lower panels) compared to lean hepatocytes (upper panels) after a 2 h-exposure to various concentrations of GCDCA (50-200 µM). **(C)** Primary fatty hepatocytes and lean hepatocytes were cultured for 4 hours and subsequently exposed to 50-200 µM GCDCA for 1 hour. The ATP concentration in these hepatocytes was measured 5 hours after isolation. **(D)** Primary fatty hepatocytes and lean hepatocytes were cultured for 4 hours and subsequently exposed to 50-200 µM GCDCA for 4 hours. The ATP concentration in these hepatocytes was measured 8 hours after isolation.

Hence, we measured total (cellular + extracellular) ATP levels, in the culture medium to determine whether the susceptibility of fatty hepatocytes to necrosis and the reduced sensitivity of fatty hepatocytes to GCDCA-induced apoptosis is caused by lower ATP level in fatty hepatocytes. Interestingly, both cultured fatty hepatocytes and lean hepatocytes had similar basal levels of ATP at 5 hours and 8 hours after isolation (Figure3C and D), suggesting that the higher susceptibility

of fatty hepatocytes to necrosis is not due to lower ATP levels in these cells. Next, we measured ATP levels in cultured hepatocytes after 1 hour and 4 hours exposure to various concentrations of GCDCA (50-200 μM). Again, there was no difference in the pattern of decline of ATP levels in both fatty and lean GCDCA-treated hepatocytes (Figure3C and D), suggesting that the reduced sensitivity of fatty hepatocytes to bile acid-induced apoptosis is not due to lower basal ATP levels or a different decline pattern in ATP during GCDCA-exposure.

Fatty hepatocytes do not show altered sensitivity to cytokine-induced apoptosis compared to lean hepatocytes

Tumor necrosis factor-α in combination with actinomycin-D induces caspase-3 activity in primary rat hepatocytes [20] that peaks at 16 h in fatty and lean hepatocytes (data not shown). We compared the sensitivity of fatty and lean hepatocytes to TNFα/ActD-induced apoptosis at this time point. The caspase-3 activity assay and acridine orange staining revealed that both types of hepatocytes are equally sensitive to cytokine-induced apoptosis and that apoptosis is the dominant mode of cell death in this model (Figure4A and B).

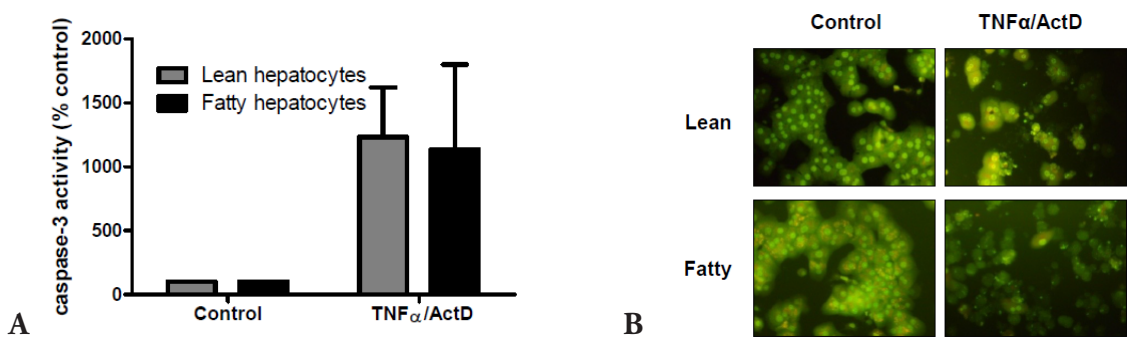

Figure4: Fatty hepatocytes do not show increased sensitivity to tumor necrosis factor-α/actinomycin-D (TNFα/ActD)-induced apoptosis. (A) Caspase-3 activity in rat hepatocytes. Primary fatty hepatocytes and lean hepatocytes were treated for 16 hours with 20 ng/ml of TNFα in the presence of 200 ng/ml of ActD. **(B)** Acridine orange staining. Treatment with TNFα/ActD induces nuclear condensation, fragmentation and cellular blebbing in fatty hepatocytes (lower panels) and lean hepatocytes (upper panels).

Discussion

In this study, we report that Cyclophilin D (Ppid) and Ant-1, the two crucial components of the mPTP, are up-regulated in fatty hepatocytes. Moreover, fatty hepatocytes appear to be more susceptible to necrosis, but show a reduced sensitivity to GCDCA-induced apoptosis compared

to lean hepatocytes even in the presence of normal ATP levels. On the other hand, they do not show an altered sensitivity to TNFα/ActD-induced apoptosis. We suggest that the up-regulation of mPTP proteins plays a critical role in the increased susceptibility of fatty hepatocytes to necrosis.

Mitochondrial dysfunction and alterations in the mPT have been suggested to play a role in the development of steatohepatitis in NAFLD [16-18]. Importantly, mPTP opening (which initiates mPT) and up-regulation of mPTP proteins are suggested to initiate necrosis and increase spontaneous cell death in vivo [9, 13, 14, 23]. However, a detailed analysis of the expression of these proteins in fatty hepatocytes has not been described before. It is important to define the primary mode of cell death in fatty hepatocytes and the underlying mechanisms in order to develop effective therapeutic strategies aiming at minimizing the incidence of hepatocyte cell death-induced liver inflammation in NAFLD. We used hepatocytes isolated from Zucker obese rats as a model of NAFLD [22]. These hepatocytes contained higher TG levels than lean Zucker hepatocytes and hence serve as a good model for fatty hepatocytes in NAFLD. Directly after isolation, the viability of fatty hepatocytes was similar to the lean hepatocytes, however, a clear increased level of necrosis was detected upon culturing of the fatty hepatocytes.

Although apoptosis and necrosis are two distinct modes of cell death, they share certain cellular and molecular features. Mitochondria are involved in both apoptosis and necrosis in hepatocytes [6, 8, 27, 28]. An important step in mitochondrial-dependent cell death is the mitochondrial permeability transition (mPT). Permeabilization triggers the release of various proteins from mitochondria into the cytosol where they activate apoptotic and/or necrotic cascades. Opening of two different mitochondrial channels has been suggested to play a role in mitochondrial permeabilization: the permeability transition pore (mPTP) in the inner membrane and the mitochondrial apoptosis-induced channel (MAC) in the outer membrane. Several studies have indicated that the mPTP is more important in necrosis than apoptosis [11, 12]. Opening of the mPTP results in non-selective diffusion of solutes across the mitochondrial inner membrane, leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation, swelling of mitochondria and eventually cell death [7-9]. Although the exact structure of the mPTP has not been elucidated yet, Cyclophilin D, ANT and VDAC have been suggested to be components of the mPTP [9, 12, 14, 23, 29]. It has been shown that cells containing higher amounts of Cyclophilin D are indeed hypersensitive to necrotic cell death [13]. In accordance, our data demonstrate that fatty hepatocytes are more susceptible to necrosis most likely due to the higher expression of Cyclophilin D. It has been shown that the ANTs are non-essential components of the mPTP. However, the ANTs do have an important role in regulating permeability transition by modulating the sensitivity of the mPTP to

[Ca $^{2+}$]-overload and ANT ligands [29]. Cyclophilin D is thought to facilitate a calcium-triggered conformational change in ANT, converting it into an open pore [11]. Therefore, the up-regulation of Ant-1 in fatty hepatocytes can also play a role in the increased susceptibility of fatty hepatocytes to necrosis. Some studies have suggested that the mPTP contains the outer membrane channel protein VDAC [30, 31]. However, a recent study in which all VDAC isoforms were knocked out have raised doubt about the involvement of outer membrane components in the mPTP as the knockouts still have a functional mPTP [32]. In other studies, VDAC oligomers were suggested to be directly responsible for cytochrome c release and apoptosis [33-35]. Our data showed an increase in mRNA expression levels of Vdac-1 and Vdac-3 in fatty hepatocytes compared to lean hepatocytes, but we were not able to detect a difference at the protein level between the two types of hepatocytes due to the lack of specificity of the available antibody for the Vdac isoforms. Whether the increase in mRNA expression level of Vdac-1 or Vdac-3 has an effect on the susceptibility of fatty hepatocytes to necrosis and/or apoptosis remains to be determined. Taken together, up-regulation of Cyclophilin D and Ant-1 conceivably may be key events in hypersensitizing fatty hepatocytes to necrosis.

It has been observed that Cyclophilin D null fibroblasts are not protected from TNFα-induced apoptosis, suggesting that cytokine-induced cell death is not dependent on Cyclophilin D and mitochondrial permeability transition [14]. This may explain why fatty hepatocytes do not show altered sensitivity to cytokine-induced apoptosis despite the up-regulation of mPTP proteins. Although the mPTP is central to necrosis, it can open transiently in response to insults such as oxidative stress and [Ca^{2+}]-overload. In these situations, the transient mPTP opening may be sufficient to cause cytochrome c release and induce apoptosis. Mice overexpressing Cyclophilin D showed increased apoptosis of heart-muscle cells in vivo. These hearts were also enlarged and contained swollen, leaky mitochondria. This suggests that excess Cyclophilin D still leads to sufficient opening of pores to cause apoptosis [11, 14]. Importantly, Cyclophilin D and mPT are crucial for [Ca^{2+}] overload-induced apoptosis and GCDCA-induced apoptosis in hepatocytes is associated with an increase in intracellular [Ca^{2+}] [14, 36]. Transient mPTP opening, despite up-regulation of mPTP proteins, may explain why fatty hepatocytes are still susceptible to bile acid-induced apoptosis, although their sensitivity is reduced even in the presence of normal ATP levels. This finding is in accordance with a previous report indicating that fatty hepatocytes may show a reduced sensitivity to GCDCA-induced apoptosis and are more susceptible to necrosis. Although the mechanisms are not completely known, alterations in mPT have been suggested to contribute to these findings [25].

Some studies have shown that treatment of fatty hepatocytes with mPTP inhibitors such as cyclosporin A (CSA), an inhibitor of cyclophilins, protects fatty hepatocytes against bile acid-induced necrosis [25, 37]. Although we have not tested these compounds in this study, such beneficial effects of mPTP inhibitors are lending indirect support to our proposed mechanism that fatty hepatocytes are vulnerable to necrosis due to increased levels of mPTP related proteins. Increased susceptibility to necrosis instead of apoptosis in fatty hepatocytes is expected to render fatty livers prone to necrosis-induced inflammation. Once inflammation occurs, the prognosis of NAFLD patients declines [1, 4]. Our study provides data regarding the mechanism underlying the increased vulnerability of fatty liver to necrosis and introduces potential targets for therapy to prevent necrosis in fatty livers namely by inhibiting mPTP in hepatocytes. Such an apporoach would protect fatty livers against inflammation and exacerbation of liver damage.

In summary, our study demonstrates that fatty hepatocytes are more susceptible to necrosis conceivably due to the increased expression of the mPTP-related proteins Cyclophilin D and ANT.

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Chapter 4

Angiotensin II protects primary rat hepatocytes against bile acid-induced apoptosis

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Submitted

Abstract

Background and Aim: Angiotensin II (AT-II) is a profibrotic compound that acts via membrane-bound receptors (AT-1R/AT-2R) and thereby activates hepatic stellate cells (HSCs). AT-II receptor antagonists are thus considered in the treatment of liver fibrosis. However, multiple case reports suggest that AT-1R receptor antagonists may induce liver injury. Therefore, we investigated the effect of AT-II and its receptors on cytokine-, oxidative stress- and bile salt-induced cell death in hepatocytes.

Methods: Primary rat hepatocytes were exposed to TNFa/Actinomycin D, the ROS-generating agent menadione or the bile salt, GCDCA, to induce apoptosis. AT-II (100 nmol/L) was added 10 minutes prior to the cell death-inducing agent. AT-1R antagonists (Sartans) and the AT-2R antagonist PD123319 were used at 1 μmol/L. Apoptosis (caspase-3 activity, acridine orange staining) and necrosis (Sytox green staining) were quantified.

Results: Expression of CHOP (marker for ER stress) and AT-II receptor mRNAs were quantified by Q-PCR. AT-II dose-dependently reduced GCDCA-induced apoptosis of hepatocytes (-50%, $p< 0.05$) without inducing necrosis. AT-II did not suppress TNF/Act-D and menadione-induced apoptosis. Only the AT-1R antagonists abolished the protective effect of AT-II against GCDCA-induced apoptosis. AT-II increased phosphorylation of ERK and a significant reversal of the protective effect of AT-II was observed when signaling kinases, including ERK, were inhibited. Moreover, AT-II prevented the GCDCA-induced expression of CHOP.

Conclusion: Angiotensin II protects hepatocytes from bile salt-induced apoptosis through a combined activation of PI3-kinase, MAPKs, PKC pathways and inhibition of bile salt-induced ER stress. Our results provide an explanation for the observed hepatotoxicity of sartans in some patients with chronic liver injury.

Introduction

Angiotensin II (AT-II) is the effector peptide of the renin angiotensin system (RAS), which plays a crucial role in regulating blood pressure. In addition to the systemic generation of AT-II in the circulation by RAS, AT-II is also produced locally in various organs, including kidney, vessels, heart, adrenal gland, brain and liver, as a result of a process commonly termed "tissue" renin-angiotensin system (RAS) (5). Tissue RAS plays an important role in maintaining cardiovascular homeostasis, but also in mediating diverse physiologic functions such as cell growth, cell differentiation and apoptosis (38). The effects of angiotensin II on organs are mediated via the AT-II type 1 and type 2 receptors (AT-1R and AT-2R) (38). It has been shown that components of the RAS are present and activated in chronic liver diseases (10, 36).

Chronic liver diseases, including cholestatic liver disease, are characterized by loss of functional liver mass due to hepatocyte cell death and the development of liver fibrosis that may progress to end-stage liver cirrhosis. Hepatic RAS is suggested to play an important role in liver fibrosis (50). Most, if not all, of the key components of RAS that lead to the generation of AT-II are present in the liver (28, 50) and are induced and/or redistributed in liver injury (10, 23, 36, 37). AT-II levels are increased both in plasma and in liver tissue in rat models of liver disease, as well as in cirrhotic patients (4, 36). It was shown that AT-II, generated by systemic RAS and/or tissue RAS, plays a role in the progression of liver fibrosis through activation and proliferation of hepatic stellate cells (HSCs) (8, 9). Moreover, activated hepatic stellate cells express RAS-components and synthesize AT-II themselves (10). HSC-derived AT-II, as well as systemic AT-II, can exert paracrine and endocrine actions on hepatocytes, which express high levels of AT-1R (10). Recent studies revealed that blocking the RAS pathway with either AT-1R blockers (ARB) or angiotensin converting enzyme inhibitors (ACEi) attenuates the progression of liver fibrosis in animal models of chronic liver diseases (16, 20, 50). Consequently, blockade of AT-II signal transduction has been suggested to be a beneficial therapy in patients with chronic liver diseases. Until now, only a small number of studies examining the effect of RAS inhibition on fibrosis in human liver diseases have been reported and there are no results from large randomized trials (reviewed in (50)). Notably, a recent cohort study in chronic hepatitis C patients with advanced liver fibrosis showed that ACEi/ARB therapy does not prevent the progression of hepatic fibrosis (1). On the other hand, there are multiple (case) reports ARBs and ACEis may induce hepatocellular injury and/or cholestasis (2, 3, 7, 13, 18, 26, 33, 41, 45, 48). Losartan and candesartan were found to induce hepatocellular injury in hypertensive patients with normal liver function tests prior to the start of the therapy (3, 7, 13,

33, 45, 48). Irbesartan therapy leads to hepatocyte cholestasis and degeneration in hypertensive patients (2, 18) and valsartan has been reported to induce lobular necrosis and inflammation in the liver (26, 41). There are also numerous reports of the potential hepatotoxicity of ACE inhibitors (reviewed in(17)). Thus, inhibition of the RAS system in fibrotic liver disease may have negative effects on liver function and hepatocyte viability in particular.

Liver injury may be caused by (a combination of) inflammation, oxidative stress and increased bile salt levels, leading to apoptosis and/or necrosis of hepatocytes. Therefore, we studied the effect of AT-II on cytokine-, ROS- and bile salt-induced apoptosis and necrosis in primary rat hepatocytes. AT-II specifically attenuated bile salt-induced apoptosis, but not cytokine- or oxidative stress-induced apoptosis. Subsequently, we analyzed the involvement of the AT-II receptors, protein kinase signaling pathways and ER stress in bile acid-induced apoptosis in detail.

Materials and Methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. All the experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

Rat hepatocyte isolation

Hepatocytes were isolated and cultured in multiwell plates (Greiner bio-one) in William's E medium in a humidified incubator at 37 ºC and 5% CO2 as described before (49).

Experimental design

Experiments were started after the attachment period of 4 hours. Monolayers of cultured primary hepatocytes were treated with angiotensin II (Sigma-Aldrich) at the indicated concentrations starting 10 minutes prior to the exposure to 50 µmol/L GCDCA (Sigma-Aldrich) for 4 hours or to 100 µmol/L of TLCS (Sigma-Aldrich) for 6 hours or to 20 ng/ml recombinant murine Tnfα (R&D

Systems, Abingdon, United Kingdom) for 16 hours or to 50 μmol/L menadione (superoxide anion donor; Sigma-Aldrich) for 9 hours unless stated otherwise. Signal transduction pathways were inhibited using 10 μmol/L of the ERK1/2 inhibitor U0126 (Promega, Madison, USA), 10 μmol/L of the p38 inhibitor SB 203580 (Calbiochem), 50 μmol/L of the PI3 kinase inhibitor LY 294002 (Calbiochem), 1 μmol/L of the protein kinase-C inhibitors Calphostin-C and Bisindolylmaleimide I (BSM-I) (Calbiochem), and 200 ng/ml of the transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, the Netherlands). Angiotensin II type 1 receptor was inhibited by the receptor antagonists Losartan (Merck), Valsartan (Novartis Pharma), Irbesartan (Bristol-Myers-Suibb) and Candesartan (Astra Zeneca, Zoetermeer, the Netherlands) all used at 1 μmol/L. Angiotensin II type 2 receptor was inhibited by 1 μmol/L of the selective antagonist PD 123319 (Sigma-Aldrich). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 minutes prior to the apoptotic stimuli unless stated otherwise. Every experimental condition was performed in triplicate wells and each experiment was repeated at least three times using hepatocytes from different rats. Cells were harvested at the indicated time points as described previously (44).

Apoptosis and necrosis assays

Caspase-3 activity was measured as described previously (44). The arbitrary fluorescence unit (AFU) was corrected for the amount of protein. Protein concentration was determined using the Bio-Rad protein assay kit. Sytox green (Invitrogen) and acridine orange (Sigma-Aldrich) were used to visualize necrotic and apoptotic cell death, respectively, as described before (54).

Quantitative PCR

RNA isolation, reverse transcription PCR and quantitative PCR (qPCR) were performed as described previously (54). Each sample was analyzed in duplicate. 18S mRNA levels were used as endogenous control. Primers and probes are listed inTable 1.

Table 1: Sequences of primers and probes used for quantitative PCR analysis

18 S Rat	Sense Antisense Probe	5'-CGGCTACCACATCCAAGGA- 3' 5'-CCAATTACAGGGCCTCGAAA-3' 5'FAM-CGCGCAAATTACCCACTCCCGA- TAMRA3'
Chop Rat	Sense Antisense Probe	5'-TCCTGTCCTCAGATGAAATTGG- 3' 5'-TCAAGAGTAGTGAAGGTTTTTGATTCT-3' 5'FAM-CACCTATATCTCATCCCCAGGAAACGAAGA- TAMRA3'
AT-1R Rat	Sense Antisense Probe	5'-GCCCAGCGGGACTCTGT- 3' 5'-CTAATGTAGATAATGTCCAGGAAGATGGT-3' 5'FAM-TTGGCATGTTTCTTGGTGGCTTGGT-TAMRA3'

Western-blot analysis

Western blot analysis was performed as described previously (54). Expression of selected protein was assessed using monoclonal mouse antibody against phosphorylated ERK1/2 (p44/42) at a dilution of 1:1000. Blots were subsequently stripped using 0.1% SDS/0.1% Tween – PBS at 65°C for 30 minutes and incubated with 1:4000 diluted monoclonal mouse antibody against GAPDH (Calbiochem, La Jolla, CA. USA). Horse radish-peroxidase conjugated rabbit anti-mouse Ig (DAKO, Denmark) was used every time as a secondary antibody at a dilution of 1:2000.

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± SD. A Student t-test or two way ANOVA test was used to determine the significance of differences between experimental groups. A P-value of less than 0.05 ($P<0.05$) was considered statistically significant.

Results

Angiotensin II inhibits bile acid-induced caspase-3 activity and apoptotic nuclear morphology

TNFα (20 ng/ml)/ActD (200 ng/ml), menadione (at 50 μM) and GCDCA (at 50 μM) induce caspase-3 activity in primary rat hepatocytes peaking after 16 hours-, 9 h- and 4h-exposure, respectively (15, 42).

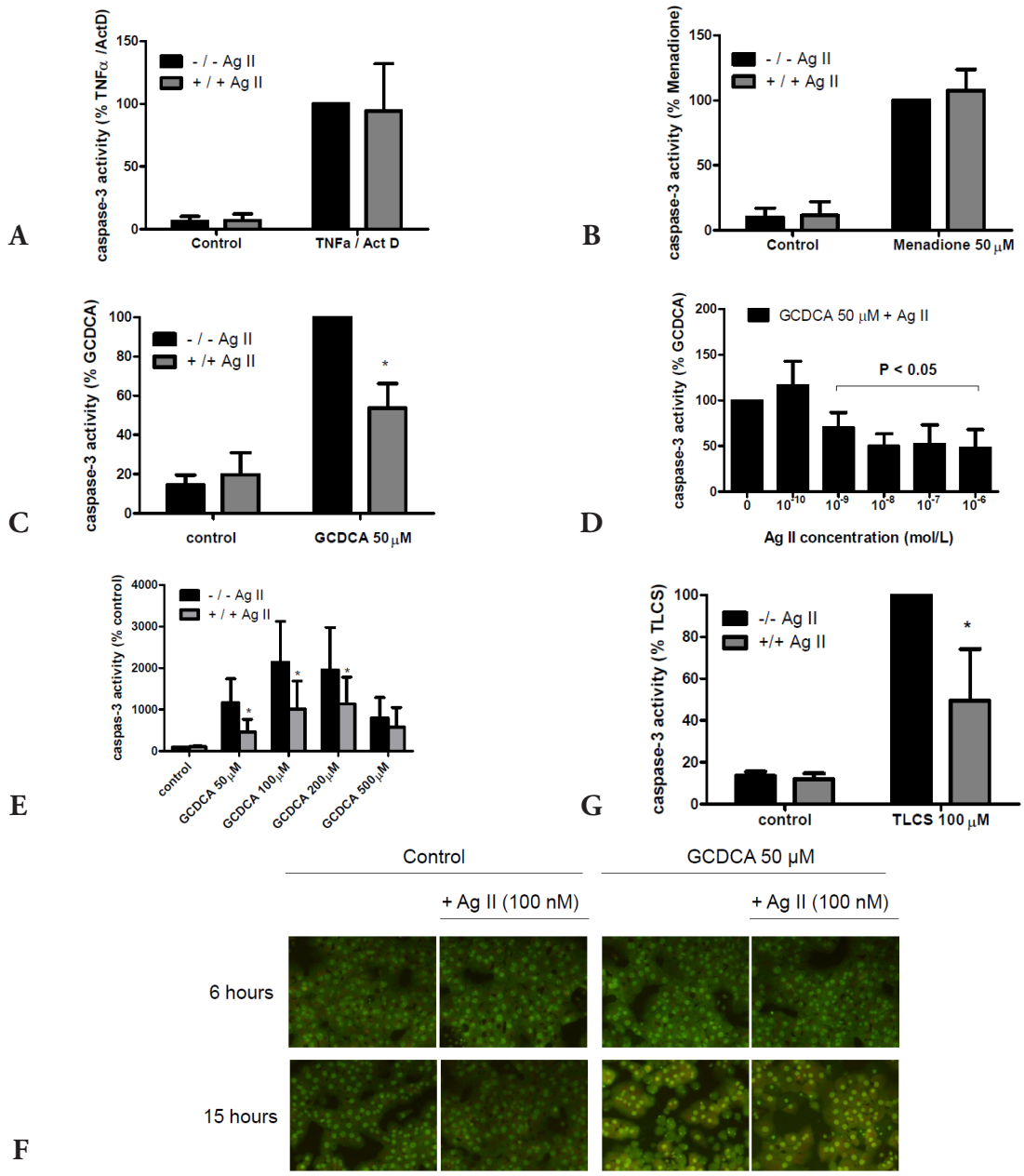


Figure1: Angiotensin II (Ag II) inhibits glycochenodeoxycholic acid (GCDCA)-induced caspase-3 activity and nuclear fragmentation in rat hepatocytes but does not protect rat hepatocytes against TNFα- and menadione-induced apoptosis. (A) Caspase-3 activity in rat hepatocytes treated with 20 ng/ml of TNFα and 200 ng/ ml of transcriptional inhibitor actinomycin-D for 16 hours in the presence and absence of 100 nmol/L Ag II. **(B)** Caspase-3 activity in rat hepatocytes treated with 50 μmol/L of menadione for 9 hours in the presence and absence of 100 nmol/L Ag II. **(C)** Primary rat hepatocytes were treated for 4 hours with 50 μmol/L of GCDCA, 100 nmol/L of Ag II or a combination of both. Ag II was added 10 min prior to the addition of GCDCA. * $P < 0.05$ for GCDCA +Ag II vs. GCDCA alone. **(D)** Hepatocytes were treated with different concentrations of Ag II 10 min prior to the addition of 50 μmol/L of GCDCA for 4 hours. **(E)** Ag II (100nmol/L) significantly inhibits GCDCA-induced caspase-3 activation. * $P < 0.05$ for GCDCA (50, 100, 200 μM) + Ag II vs GCDCA (50, 100, 200μM) alone. **(F)** Acridine orange staining. Treatment with 50 μmol/L of GCDCA induces nuclear condensation and fragmentation which is blocked with 100 nmol/L Ag II at 15 hours-time point. **(G)** Ag II inhibits tauro-lithocholic acid-3 sulfate (TLCS)-induced caspase-3 activity. Hepatocytes were treated for 6 hours with 100 μmol/L of TLCS, 100 nmol/L of Ag II or a combination of both. Cells were treated with Ag II 10 min prior to the addition of TLCS. * $P < 0.05$ for TLCS +Ag II vs. TLCS alone.

The effect of AT-II on cytokine-, oxidative stress- and bile acid-induced caspase-3 activity in primary hepatocytes was investigated at these time points. Exposure of primary rat hepatocytes to AT-II (100 nmol/L) alone did not induce caspase-3 activity (Figure1A), nor did it reduce the caspase-3 activity in TNFα/ActD-treated or menadione-treated rat hepatocytes (Figure1A and B). In contrast, AT-II significantly inhibited the GCDCA-induced caspase-3 activity (Figure1C; -50% approximately, $p<0.05$), which was concentration-dependent (Figure1D). In all subsequent experiments a concentration of 10^{-7} mol/L (100 nmol/L) of AT-II was used. AT-II attenuated the GCDCA-induced caspase-3 activity at different apoptosis-inducing concentrations of GCDCA (50-200 μM; Figure1E). At higher GCDCA concentrations the primary mode of cell death shifts to necrosis (54), which is not prevented by AT-II (Figure1E). AT-II may delay, rather than prevent GCDCA-induced apoptosis in rat hepatocytes. To establish true protection by AT-II, GCDCA-treated hepatocytes were stained with acridine orange after 6 and 15 h exposure with and without AT-II. Nuclear fragmentation and condensation, markers for end-stage apoptosis, became detectable 6 hours after the addition of GCDCA and increased over time (shown for 15 h treatment;Figure1F). Nuclear fragmentation and condensation were reversed to control levels when GCDCA-exposed hepatocytes (after 15h) were co-treated with AT-II (Figure1F). Importantly, AT-II did not induce necrosis in hepatocytes, nor did it increase the number of necrotic cells after exposure to GCDCA (data are not shown).

To determine whether AT-II is also protective towards cytotoxic effects of other bile salts, the effect on tauro-lithocholic acid-3 sulfate (TLCS)-induced caspase-3 activity was studied. Similar as for GCDCA, AT-II attenuated TLCS-induced caspase-3 activity in primary rat hepatocytes with approximately 50% (Figure1G).

Remarkably, no protective effect of AT-II was detected in bile salt (GCDCA)- and cytokine-induced apoptosis in HepG2-rNtcp cells (Suppl. Fig.1 A and B), which suggests that the AT-II-stimulated signaling pathways are absent in these cells.

The protective effect of angiotensin II is mediated via the angiotensin receptor type -1

AT-II signaling depends on the presence of AT-II receptors in cellular membranes. AT-1R but not AT-2R has previously been shown to be expressed in hepatocytes (36). We confirmed significant mRNA expression for AT-1R in rat hepatocytes, comparable to those in activated rat hepatic stellate cells, used as positive control (data are not shown). The role of AT-1R and AT-2R in the protective

effect of AT-II against GCDCA-induced apoptosis was investigated using specific antagonists of AT-1R (various sartans) or AT-2R (PD123319).

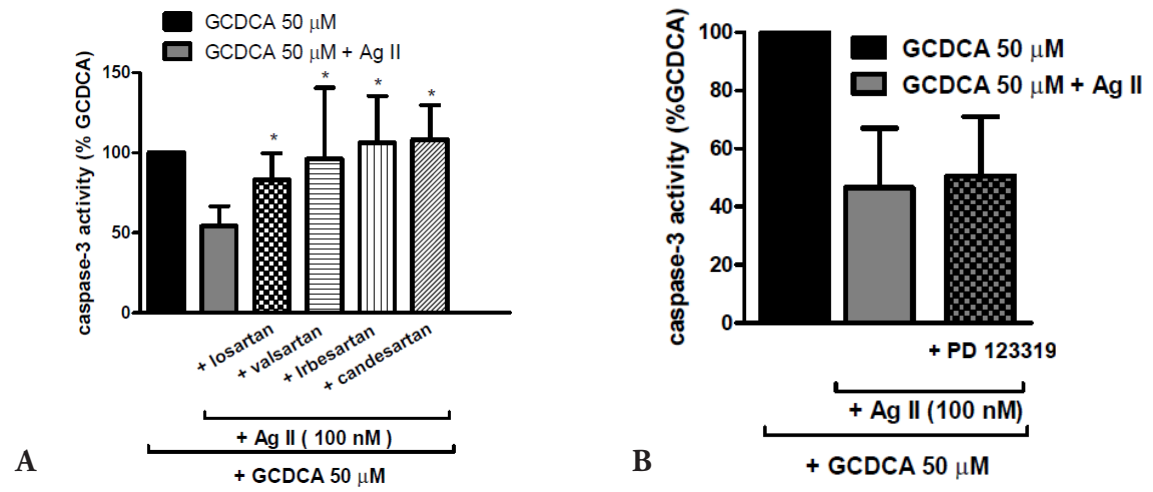


Figure2: The protective effect of angiotensin II (Ag II) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis is mediated via the angiotensin II type-1 receptor (AT-1R). (A) Hepatocytes were treated with 1 µmol/L of selective AT-1R antagonists (Losartan, Valsartan, Irbesartan and Candesartan) 30 minutes prior to the addition of GCDCA (50 µM) + Ag II (100 nmol/L). * $P < 0.05$ for GCDCA+ Ag II+ AT-1 antagonists vs. GCDCA + Ag II. **(B)** Hepatocytes were treated with 1 µmol/L of the selective AT-2 antagonist (PD 123319) 30 minutes prior to the addition of GCDCA (50 µM) + Ag II (100nmol/L).

The protective effect of AT-II against GCDCA-induced apoptosis was completely abolished in the presence of the various sartans tested (losartan, valsartan, irbesartan and candesartan), strongly indicating that the protective effect of AT-II is mediated through AT-1R (Figure2A). In contrast, inhibition of AT-2R with PD123319 did not affect the protective action of AT-II against GCDCA-induced apoptosis in hepatocytes (Figure2B).

Time window of anti-apoptotic action of angiotensin II

We next investigated whether AT-II needs to be present during the GCDCA treatment to protect hepatocytes against apoptosis. Hepatocytes were pre-incubated with AT-II for 10 minutes. Medium was removed and cells washed and then exposed to GCDCA in fresh medium with or without AT-II for 4 hours. Caspase-3 activity was inhibited in the presence of AT-II and the protective effect of AT-II persisted in hepatocytes exposed to GCDCA in fresh medium without AT-II (Figure3A). Furthermore, addition of AT-II up to 30 minutes after the start of the GCDCA treatment still exerted maximum protection against GCDCA-induced apoptosis (Figure3B). No protective effect of AT-II was detected when AT-II was added at later time points (1-3 h after GCDCA treatment; Figure3B). These data show that the anti-apoptotic actions of AT-II are rapidly induced and suggest

the involvement of protein kinase signal transduction pathways in the protective actions of AT-II against GCDCA-induced apoptosis.

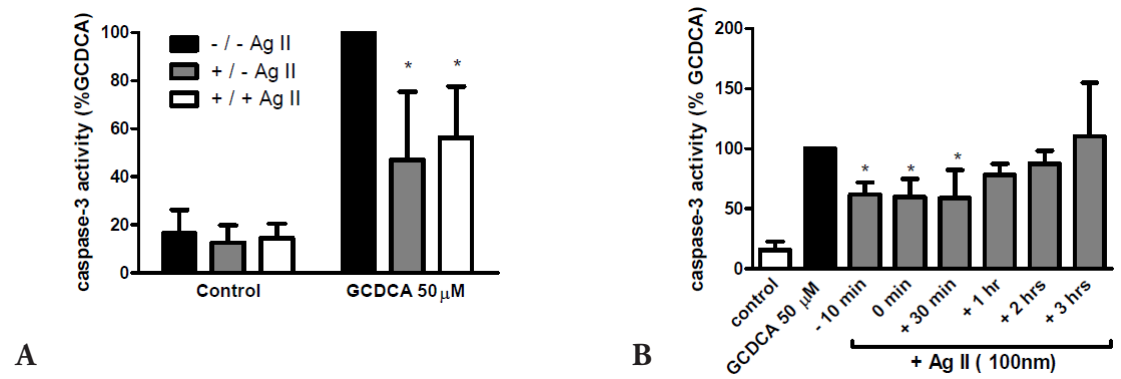


Figure3: The protective effects of angiotensin II (Ag II) are induced rapidly. (A) Hepatocytes were pre-incubated with 100 nmol/L of Ag II for 10 minutes after which cells were washed and exposed to 50 μmol/L of GCDCA alone (+ / - Ag II) or with simultaneous addition of Ag II (+ / + Ag II). * P < 0.05 for GCDCA + Ag II (+ / -) and GCDCA + Ag II (+ / +) vs. GCDCA 50 μM. **(B)** hepatocytes were stimulated with 50 μmol/L GCDCA for 4 hours (GCDCA 50). Ag II (100 nmol/L) was added 10 minutes prior to (-10 min), simultaneous with (0 min) or 30 minutes (+30 min), 1 hour (+1 hr), 2 hours (+2 hrs), 3 hours (+3hrs) after the addition of GCDCA.* P <0.05 for -10 min of Ag II, 0 min of Ag II, +30 min of Ag II vs GCDCA alone.

Anti-apoptotic action of angiotensin II depends on the combined activation of ERK1/2, p38 MAP Kinase, PI3kinase and protein kinase C

To investigate whether specific kinase pathways are involved in the anti-apoptotic effects of AT-II, GCDCA-exposed rat hepatocytes were co-treated with inhibitors of MAPK, PI3K and PKC. These inhibitors alone do not induce caspase-3 activity in rat hepatocytes, nor do they enhance the GCDCA-induced caspase-3 activation. (Figure4A). The protective effect of AT-II against GCDCA-induced apoptosis was significantly, albeit partially, abolished by inhibition of ERK1/2, p38 MAP kinases and PI3 kinase pathways (Figure4A). Additive effects were observed when ERK1/2 and p38 MAK kinase were inhibited simultaneously. In support, AT-II increased the levels of phosphorylated ERK1/2 MAPK in control hepatocytes as well as in the presence of GCDCA, which was abolished by the AT-1R antagonist candesartan (Figure4B). In the presence of PKC inhibitors, the protective effect of angiotensin II against GCDCA-induced caspase-3 activation was also abolished significantly (Figure4A).

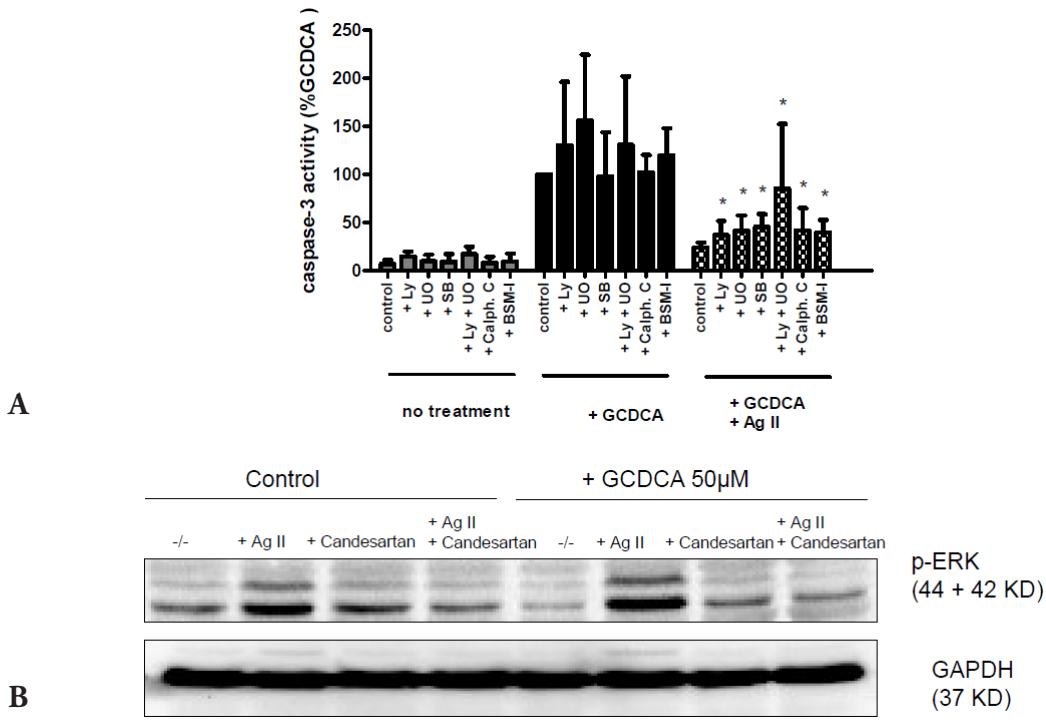


Figure4: The protective effect of angiotensin II (Ag II) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis is dependent on the activation of p38 MAP kinase, ERK1/2 MAPK, PI3K pathway and PKC pathway. **(A)** Caspase-3 activity in rat hepatocytes treated with 50 µmol/L of GCDCA in the presence and absence of 100 nmol/L Ag II and with or without the inhibitors of ERK MAPK (10 µmol/L of U0126; U0), p38 MAPK (10 µmol/L of SB 203580; SB), PI3K (50 µmol/L of LY 294002; LY) and PKC (1 µmol/L of Calph. C or BSM-I). *P < 0.05 for GCDCA + Ag II+ LY vs. GCDCA+ Ag II , for GCDCA + Ag II+ SB vs. GCDCA+ Ag II, for GCDCA+ Ag II + U0 vs. GCDCA+ Ag II and for GCDCA+ Ag II + U0+LY vs. GCDCA+ Ag II, for GCDCA+ Ag II + Calph.C vs. GCDCA + Ag II and for GCDCA + Ag II + BSM-I vs. GCDCA + Ag II. **(B)** Western blot analysis for phosphorylated ERK1/2 MAPK in cell lysates of GCDCA-exposed hepatocytes (10 min) with and without Ag II. ERK1/2 MAPK phosphorylation was inhibited in the presence of AT-1 antagonist (1 µmol/L of candesartan).

Angiotensin II attenuates GCDCA-induced endoplasmic reticulum stress

Besides kinase signaling pathways, hydrophobic bile acids, such as GCDCA, may also induce apoptosis by increasing ER stress and induction of the unfolded protein response via transcription factors like CHOP (12, 47). Induction of CHOP is a sensitive marker for ER stress and CHOP-knockout mice are protected against cholestasis-induced hepatocyte apoptosis (25, 46). Indeed, GCDCA treatment of rat hepatocytes lead to a transient increase in CHOP mRNA levels that peaked at 2 hours after GCDCA treatment, after which they gradually decrease to control levels after 4-8 hours (Figure5). AT-II prevented the GCDCA-induced expression of CHOP in rat hepatocytes at all different time points tested, while it did not exert any effect on CHOP levels in control hepatocytes (Figure5).

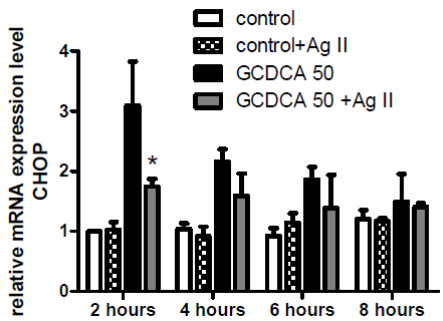


Figure5: Angiotensin II (Ag II) attenuates GCDCA-induced ER stress in rat hepatocytes. QPCR analysis of Chop mRNA (ER stress marker) in rat hepatocytes treated with 50 µmol/L GCDCA at several time points with or without 100 nmol/L of Ag II using cDNA of hepatocytes. mRNA expression level is presented relative to control. * $P < 0.05$ for GCDCA +Ag II vs. GCDCA alone.

Discussion

In this study, we report that angiotensin II (AT-II) protects primary rat hepatocytes specifically against bile salt-induced apoptosis. We demonstrate that the protective effects of AT-II are mediated via AT-1R signal transduction and activation of ERK, p38 MAPK, PI3K and PKC signaling pathways. In addition, we show that AT-II treatment reduces the bile salt-induced expression of CHOP, an ER stress-induced effector peptide, in primary rat hepatocytes. These findings suggest that inhibition of systemic and/or tissue RAS pathway in (cholestatic) liver fibrosis should be monitored carefully as it blocks the hepatoprotective action of AT-II on liver parenchymal cells.

AT-II antagonists (ACEi and/or ARB) are considered as therapeutics for fibrotic liver diseases as they prevent HSC activation and proliferation (50). However, the effect of AT-II antagonist therapy on hepatocytes has not previously been studied. Hepatocytes are the most abundant cell type in the liver. They express several components of RAS including AT-1R and exhibit numerous responses to AT-II, including the activation of gene transcription (14, 31, 36, 53). Therefore, it is likely that AT-II harbors specific roles in (diseased) hepatocytes and AT-II antagonist therapy may lead to hepatotoxicity. Indeed, ACEi- and ARB-induced hepatocellular injury and liver damage has been reported previously (3, 7, 13, 33, 45, 48). Losartan, Candesartan and Irbesartan were found to induce hepatocellular injury, hepatocyte cholestasis and degeneration in patients with essential hypertension and normal liver function tests prior to the start of the therapy (2, 18). Valsartan was found to induce lobular necrosis and inflammation in the liver (26, 41) and ACE inhibitors were reported to cause severe hepatic necrosis or drug-induced hepatotoxicity (reviewed in (17)).

Several mechanisms have been suggested for ARB-induced hepatotoxicity and cholestasis such as metabolic idiosyncrasy and immune mechanisms. However, the hypothesis that angiotensin II has hepatoprotective effects and ARBs sensitize hepatocytes to toxic stimuli has not been investigated before. In the present study, we have used several apoptotic stimuli (cytokines, ROS and bile salts) that are increased in chronic liver diseases and lead to hepatocyte injury, to investigate the effect of AT-II on hepatocytes. Our data strongly demonstrate that AT-II has an AT-1R-mediated hepatoprotective effect on hepatocytes which are prevented by several ARBs. It has previously been observed that hepatocytes express AT-1R and the up-regulation of this receptor in cholestatic liver injury has been reported, whereas the AT-2R is suggested to be absent or very low expressed in hepatocytes (10, 43). Our data confirm that AT-2R-mediated signaling does not play a major role in hepatocytes cell death or survival.

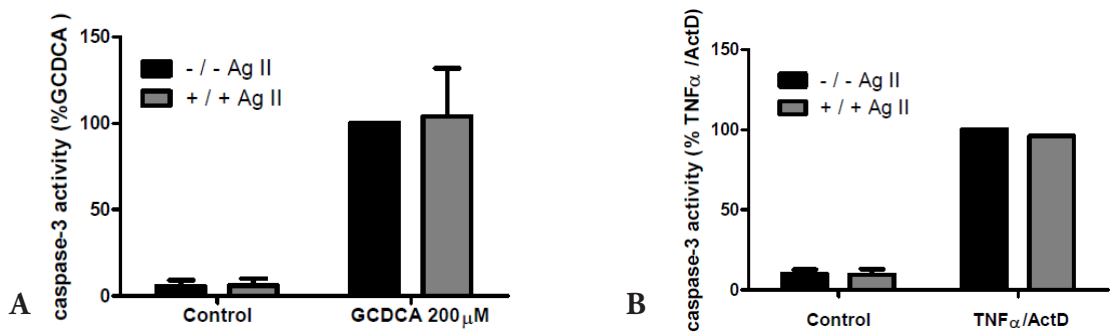
Interestingly, AT-II protects hepatocytes against bile salt-induced apoptosis, but not against TNFα- or menadione-induced apoptosis. This could be due to the induction of different apoptotic signaling pathways in response to these apoptotic stimuli. Menadione induces apoptosis in hepatocytes via production of reactive oxygen species (ROS) leading to oxidative stress in hepatocytes (15). Previously, we have shown that ROS are not essential for bile salt-induced cell death in hepatocytes and anti-oxidant therapy does not protect hepatocytes against bile salt-induced apoptosis (54), indicating different mechanisms for bile acid and ROS-induced hepatocyte apoptosis. TNFα-induced apoptosis in hepatocytes is shown to be mediated via death-receptor-dependent signaling (TNF receptor type 1, TNF-R1), leading to recruitment of Fas associated death domain (FADD), activation of caspase-8 and pro-apoptotic proteins, Bak and Bid (43, 44). In contrast, it has been shown that bile salt-induced caspase-8 activation is FADD-independent and inhibition of caspase-8 has no effect on bile salt- induced caspase-3 activation (43). This may explain why AT-II-induced signaling pathways cannot counteract the menadione- and TNFα-induced apoptotic signaling, whereas they do protect hepatocytes against bile salt-induced apoptosis.

We provide evidence that the protective effects of AT-II are mediated via a combined activation of protein kinase signal transduction pathways, as we observed partial effects of specific kinase inhibitors, reversing the protective effects of AT-II. In accordance with our data, previous studies have also indicated that AT-II stimulates activation of ERK, PI3K, p38 MAPK and PKC (11, 19, 32, 51, 52, 57). It has been observed that taurine-conjugated UDCA (TUDCA)-induced activation of the ERK, PI3K and p38 MAPK protects primary rat hepatocytes against bile salt-induced apoptosis (42). Thus, it is likely that AT-II mediated activation of these protein kinases aid to the survival of cholestatic hepatocytes. PKC signaling cascade is suggested to play an important role

in AT-II related pathophysiology (19, 58). Interestingly, PKC-alpha has been reported to inhibit hepatic apoptosis through an increase of anti-apoptotic Bcl-2 family proteins such as Bcl-XL (21, 24). Induction of the expression of anti-apoptotic Bcl-XL as a consequence of PKC activation by AT-II may be part of the hepatoprotective effect of AT-II in cholestatic hepatocytes. In addition, AT-II attenuated the bile salt-induced CHOP expression. CHOP is one of the most sensitive markers for ER stress that activates the ER stress-induced apoptotic pathway (35). Overexpression or microinjection of CHOP protein have been reported to lead to cell cycle arrest and/or apoptosis (6, 29). CHOP can induce the expression of the proapoptotic BH3-only protein Bim and the cell surface death receptor TRAIL receptor 2 (also known as death-receptor 5, DR5) and inhibit transcription of the anti-apoptotic protein Bcl-2 (30, 40, 56). In contrast, CHOP disruption in cells leads to reduced apoptosis in response to ER-stress (34). Indeed, CHOP deficiency reduces cholestasis-induced hepatocyte apoptosis (46). Activation of ERK 1/2 MAP kinase and PI3K/Akt pathway were found to attenuate the ER stress-induced apoptosis and reduce CHOP expression, leading to cell survival (22, 39, 59). Furthermore, activation of PKC signaling pathway has been suggested to inhibit the ER stress-induced caspase-12 and caspase-3 activation and therefore inhibit apoptosis (27). Interestingly, inhibition of the ER-stress response by TUDCA is a hepatoprotective mechanism in liver disease (55). Our data suggest that activation of these protein kinases in AT-II-treated hepatocytes inhibits the ER stress response, leading to hepatocyte survival.

The hepatoprotective effect of AT-II against bile salt-induced apoptosis has great clinical relevance in AT-II antagonist therapy of patients with cholestatic liver fibrosis. As patients with chronic liver diseases usually visit the clinicians when their disease is already advanced and the therapy has to start in already diseased livers containing diseased (e.g. cholestatic) hepatocytes, it is important to clarify the response of hepatocytes to therapy (AT-II vs ARBs). Our data suggest that despite the beneficial effects of ARBs on liver fibrosis, they may hypersensitize the adjacent hepatocytes to certain toxic stimuli such as bile salts, leading to further loss of functional hepatocytes. Therefore, liver function should be closely monitored during the AT-II antagonist therapy of cholestatic liver fibrosis, especially in the early stages of cholestatic liver disease (when there is still considerable amount of functional hepatocytes). In addition, targeting AT-II antagonists specifically to hepatic stellate cells can be a good solution in order to prevent hepatocyte loss during the treatment of liver fibrosis. Combined UDCA/ARBs therapy could serve as an alternative option to protect hepatocytes while treating liver fibrosis in cholestatic liver diseases, as UDCA has similar hepatoprotective effects as were observed for AT-II in our study and UDCA is already used in clinic for patients with chronic cholestatic diseases. In summary, we show that AT-II protects primary rat hepatocytes against bile

salt-induced apoptosis via the AT-1R-mediated activation of the signaling protein kinases and reduction of the ER stress-induced apoptosis in hepatocytes.



Suppl. Fig.1: Angiotensin II (Ag II) has no protective effect in bile salt- and TNFα/ActD-induced apoptosis in HepG2-rNtcp cells. The human hepatoma cell line HepG2 stably expressing the bile salt importer rNtcp, were cultured. (A) Cells were incubated with 200 μmol/L of GCDCA for 4 hours in the presence and absence of 100 nmol/L of Ag II. Cells were harvested hypotonic cell lysis buffer and caspase-3 activity was measured**. (B)** Cells were incubated with 20 ng/ml of human recombinant TNFα for 16 hours in the presence and absence of 100 nmol/L of Ag II. 200 ng/ ml of transcriptional inhibitor actinomycin-D was added to the cells prior to the addition of TNFα. Cells were harvested in hypotonic cell lysis buffer and caspase-3 activity was measured.

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Chapter 5

Pertussis toxin, an inhibitor of Gαi proteins, inhibits bile acid- and cytokine-induced apoptosis in primary rat hepatocytes

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under revision

Abstract

Background and Aim: Excessive hepatocyte apoptosis is a common event in acute and chronic liver diseases leading to loss of functional liver tissue. Approaches to prevent apoptosis have therefore high potential for the treatment of liver disease. G-protein coupled receptors (GPCR) play crucial roles in cell fate (proliferation, cell death) and act through heterotrimeric G-proteins. GαiPCRs have been shown to regulate lipoapoptosis in hepatocytes, but their role in inflammation- or bile acid-induced apoptosis is unknown. Here, we analyzed the effect of inhibiting GαiPCRs function, using pertussis toxin (PT), on bile acid- and cytokine-induced apoptosis in hepatocytes.

Methods: Primary rat hepatocytes or HepG2-rNtcp cells (human hepatocellular carcinoma cells) were exposed to glycochenodeoxycholic acid (GCDCA) or tumor necrosis factor-α (TNFα)/ actinomycin D (ActD). PT (50-200 nmol/L) was added 30 minutes prior to the apoptotic stimulus. Apoptosis (caspase-3 activity, acridine orange staining) and necrosis (sytox green staining) were assessed.

Results: PT significantly reduced GCDCA- and TNFα/ActD-induced apoptosis in rat hepatocytes (-60%, $p< 0.05$) in a dose-dependent manner (with no shift to necrosis), but not in HepG2-rNtcp cells. The protective effect of pertussis toxin was independent of the activation of selected cell survival signal transduction pathways, including ERK, p38 MAPK, PI3K and PKC pathways, as specific protein kinase inhibitors did not reverse the protective effects of pertussis toxin in GCDCA-exposed hepatocytes.

Conclusion: Pertussis toxin, an inhibitor of GαiPCRs, protects hepatocytes, but not hepatocellular carcinoma cells, against bile acid- and cytokine-induced apoptosis and has therapeutic potential as primary hepatoprotective drug, as well as adjuvant in anti-cancer therapy.

Introduction

In chronic and acute liver diseases, the liver is exposed to increased levels of cytokines, reactive oxygen species and bile acids, all of which independently can lead to loss of functional liver mass due to hepatocyte cell death. Concomitantly, hepatic stellate cells become activated, start proliferating and produce excessive amounts of extracellular matrix proteins leading to liver fibrosis, which may progress to end-stage liver disease [1]. Hepatocyte cell death can occur via apoptosis, necrosis or a combination of these different types of cell death [2]. Apoptosis is an energy-dependent process, resulting in the formation of apoptotic bodies. Apoptotic bodies are cleared by surrounding phagocytosing cells that minimize inflammation. In contrast, uncontrolled apoptosis and (secondary) necrosis trigger inflammation in the liver [3, 4]. Despite worldwide efforts to establish therapeutic strategies for liver injury, end-stage liver disease remains a high burden for public health due to the lack of effective treatments. Excessive hepatocyte apoptosis is often observed in liver disease and, as this is a highly controlled cellular mechanism, drugs and therapeutic strategies to prevent hepatocyte apoptosis may help to maintain sufficient liver mass and function [1].

Recently, G-protein coupled receptors (GPCRs) have been suggested as new drug targets to treat cardiac diseases and cancer, as GPCRs play crucial roles in the regulation of cell proliferation, angiogenesis, cell survival and apoptosis [5, 6]. GPCRs are the largest family of membrane proteins and are essential nodes of communication between the internal and external environment of the cells. Over 300 GPCRs have been reported in human and rodents [7]. Upon activation by agonists, GPCRs activate heterotrimeric G-proteins ($G\alpha\beta\gamma$). These subunits subsequently activate second messengers (e.g. cAMP, Ca^{2+} and protein kinases), submitting the GPCR induced-signal to the intracellular targets. Heterotrimeric G-proteins are divided into 4 families (i.e., $G\alpha s$, $G\alpha i$, $G\alpha q/11$ and $G\alpha 12/13$) based on the $G\alpha$ subunit sequence identity and signaling activity [8].

A number of bacterial endotoxins are suggested as excellent tools to study the function of GPCRs, as they covalently modify the α-subunit of G-proteins, altering their function (reviewed in [8]). Pertussis toxin (PT), an exotoxin produced by Bordetella pertussis (the causative agent of whooping cough), is shown to be a mono-ADP-ribosyltransferase that covalently modifies the α-subunit of Gi proteins. This ribosylation is irreversible and prevents the G-proteins from interacting with G protein-coupled receptors on the cell membrane, thus interfering with intracellular communication [9-11]. As a result, the function of effector proteins, such as adenylyl cyclase, ERK/MAPK and Ca^{2+} channels is changed and modulates cell proliferation, survival and angiogenesis [8]. Interestingly,

GPCRs antagonists have shown excellent therapeutic benefits in clinical trials in controlling tumor growth and apoptosis [6]. Therefore, GPCR-based drugs may also show therapeutic benefits in regulation of apoptosis and/or survival in liver diseases.

GPCRs are present in hepatocytes and play an important role in the regulation of several hepatocyte functions, including gluconeogenesis and lipid storage [12-15]. In addition, lysophosphatidylcholine has been shown to act via a GαiPCR-dependent mechanism in lipoapoptosis of hepatocytes [14]. Whether PT-sensitive GPCRs also play a role in other apoptotic signals, like bile acid- or cytokine-induced apoptosis, is not known. In this study, we have investigated the effect of PT-mediated inhibition of GPCRs in models of TNFα- and GCDCA-induced apoptosis in primary rat hepatocytes. Our data provide new information about the function of PT-sensitive GPCRs during liver injury and suggest new targets for treatment of liver diseases.

Materials and Methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. All the experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

Rat hepatocyte isolation

Hepatocytes were isolated and cultured in multiwell plates (Greiner bio-one) in William's E medium in a humidified incubator at 37 ºC and 5% CO2 as described before [16].

Experimental design

Experiments were started after the attachment period of 4 hours. In order to inhibit the α-subunit of G-proteins, monolayers of cultured primary hepatocytes were treated with pertussis toxin (Calbiochem, VWR International, Amsterdam, the Netherlands) at the indicated concentrations starting 30 minutes prior to the exposure to 50 μmol/L GCDCA (Sigma-Aldrich) for 4 hours or 20

ng/ml recombinant murine TNFα (R&D Systems, Abingdon, United Kingdom) for the indicated time period, unless stated otherwise. Signal transduction pathways were inhibited using 10 μmol/L of the ERK1/2 inhibitor U0126 (Promega, Madison, USA), 10 μmol/L of the p38 inhibitor SB 203580 (Calbiochem), 50 μmol/L of the PI3 kinase inhibitor LY 294002 (Calbiochem), 1 μmol/L of the protein kinase-C inhibitors Calphostin-C and Bisindolylmaleimide I (BSM-I) (Calbiochem), and 200 ng/ml of the transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, the Netherlands). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 minutes prior to the apoptotic stimuli unless stated otherwise. Each experimental condition was performed in triplicate. Each experiment was repeated at least three times using hepatocytes from different rats. Cells were harvested at the indicated time points as described previously [16].

HepG2-rNtcp cell experiments

The human hepatoma cell line HepG2 stably expressing the bile acid importer rNtcp, was cultured as described before [17]. Cells were incubated with GCDCA (200 μmol/L) or human recombinant TNFα (20 ng/ml) for the indicated time points. The transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, the Netherlands) was added to the cells prior to the addition of TNFα. Cells were harvested at the indicated time points in hypotonic cell lysis buffer [16].

Apoptosis and necrosis assays

Caspase-3 activity was measured as described previously [16]. The arbitrary fluorescence unit (AFU) was corrected for the amount of protein. Protein concentration was determined using the Bio-Rad protein assay kit. Sytox green (Invitrogen) and acridine orange (Sigma-Aldrich) were used to visualize necrotic and apoptotic cell death, respectively, as described before [18].

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± SD. A two way ANOVA test was used to determine the significance of differences between experimental groups. A P-value of less than 0.05 ($P<0.05$) was considered statistically significant.

Results

Pertussis toxin inhibits bile acid-induced caspase-3 activity and apoptotic nuclear morphology

GCDCA (at 50 µM) induces caspase-3 activity in primary rat hepatocytes that peaks after 4 hours exposure [16].

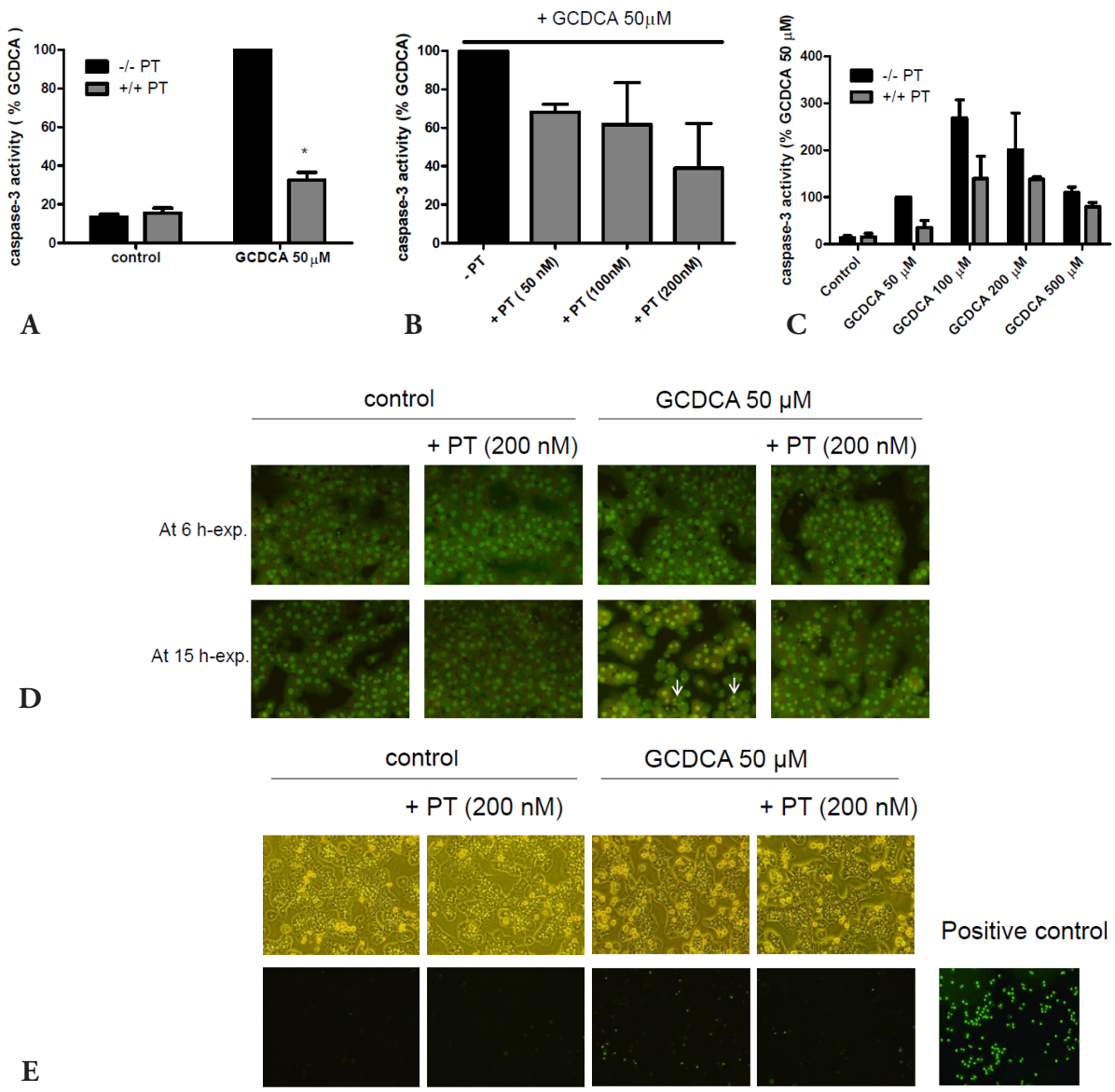

Figure1: Pertussis toxin (PT) inhibits glycochenodeoxycholic acid (GCDCA)-induced caspase-3 activity and nuclear fragmentation. (A) Primary rat hepatocytes were treated for 4 hours with 50 µmol/L of GCDCA, 200 nmol/L of PT or a combination of both. PT was added 30 min prior to the addition of GCDCA. * $P < 0.05$ for GCDCA + PT vs. GCDCA alone. **(B)** Hepatocytes were treated with different concentrations of PT 30 min prior to the addition of 50 µmol/L of GCDCA for 4 hours. **(C)** PT (200nmol/L) significantly inhibits GCDCA-induced caspase-3 activation. * $P < 0.05$ for GCDCA (50, 100, 200 µM) + PT vs GCDCA (50, 100, 200 µM) alone. **(D)** Acridine orange staining. Treatment with 50 µmol/L of GCDCA induces nuclear condensation and fragmentation which is blocked with 200 nmol/L PT at 15 hours after the addition of GCDCA. (E) Sytox green staining. PT does not induce necrosis in hepatocytes after 15 hours. Hepatocytes treated with 5 mmol/L H2 O2 were used as positive control.

The effect of PT on GCDCA-induced caspase-3 activity in primary rat hepatocytes was investigated at this time point. Exposure of primary rat hepatocytes to PT (200 nmol/L) alone did not induce caspase-3 activation in hepatocytes (Figure1 A), but significantly inhibited GCDCA-induced caspase-3 activity in a dose-dependent manner, with maximum inhibition observed at 200 nmol/L PT (-60%, $P<0.05$; Figure1 A and B). In all subsequent experiments a concentration of 200 nmol/L PT was used. PT attenuated the GCDCA-induced caspase-3 activity at different apoptotic concentrations of GCDCA (50-200 μM; Figure1 C). At higher GCDCA concentrations the primary mode of cell death shifts to necrosis [18]. PT may delay, rather than prevent GCDCA-induced apoptosis in rat hepatocytes. To establish whether PT prevents, as opposed to delaying apoptosis, GCDCA-treated hepatocytes were stained with acridine orange after 6 and 15 hours exposure with and without PT. Nuclear fragmentation and condensation, markers for end-stage apoptosis, were hardly detectable 6 hours after the addition of GCDCA, but clearly increased over time (shown for 15 hours exposure; Figure1D). The formation of fragmented and condensed nuclei was inhibited when GCDCA-exposed hepatocytes (both after 6 and 15 h) were co-treated with PT, comparable to control levels (Figure1D). Importantly, PT alone did not induce necrosis in hepatocytes, nor did it increase the number of necrotic cells after exposure to GCDCA (Figure1E).

Time window of anti-apoptotic action of pertussis toxin

PT irreversibly inhibits Gαi-signaling. If Gαi function is required for bile acid-induced apoptosis, pre-incubation with PT alone should also lead to inhibition of GCDCA-induced apoptosis in hepatocytes. Hepatocytes were pre-incubated with PT for 15 hours, which results in complete ADP-ribosylation of Gαi proteins [19]. Medium was removed and cells were washed and then exposed to GCDCA in fresh medium with or without PT for 4 hours. Caspase-3 activity was inhibited in the presence of PT and the protective effect of PT persisted in hepatocytes exposed to GCDCA in fresh medium without PT (Figure2A). These data show that the anti-apoptotic effect of PT is sustained in hepatocytes, suggesting that the protective effect of PT is mediated via PT-catalyzed irreversible ribosylation of the α-subunit preventing the G-proteins from interacting with GPCRs [11, 20]. Furthermore, addition of PT up to one hour after the start of the of GCDCA treatment still exerted maximum protection against GCDCA-induced apoptosis (Figure2B). No protective effect of PT was detected when PT was added at later time points (2-3 h after GCDCA treatment; Figure2B). These data indicate that the anti-apoptotic effect of PT is rapidly induced in

hepatocytes and suggest the involvement of Gαi protein signaling pathways in the protective action of PT against GCDCA-induced apoptosis [19].

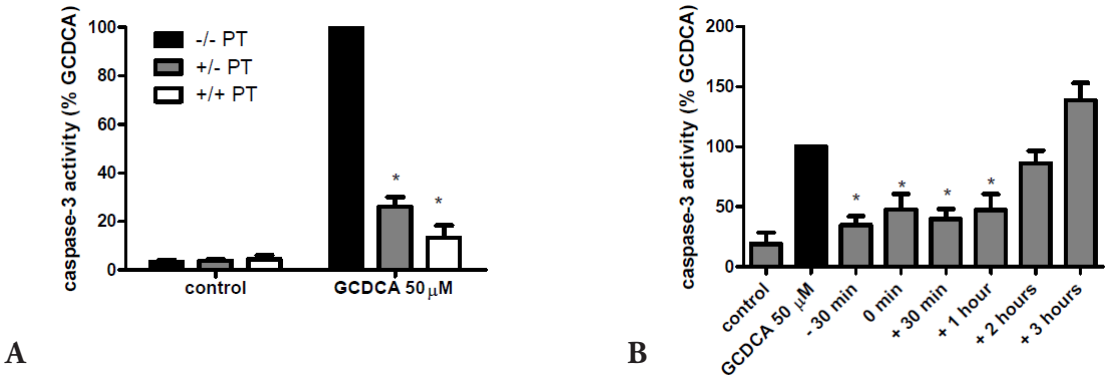

Figure2: The protective effect of pertussis toxin (PT) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis is mediated through inhibition of Gαi –proteins. (A) Hepatocytes were pre-incubated with 200 nmol/L of PT for 15 hours after which cells were washed and exposed to 50 μmol/L of GCDCA alone (+/- PT) or with simultaneous addition of PT (+/+ PT). * P < 0.05 for GCDCA + PT (+ / -) and GCDCA + PT (+ / +) vs. GCDCA 50 μM. **(B)** hepatocytes were stimulated with 50 μmol/L GCDCA for 4 hours (GCDCA 50 μM). PT (200 nmol/L) was added 30 minutes prior to (-30 min), simultaneous with (0 min) or 30 minutes (+30 min), 1 hour (+1 hr), 2 hours (+2 hrs), 3 hours (+3hrs) after the addition of GCDCA.* P <0.05 for -30 min of PT, 0 min of PT, +30 min of PT, +1 hr of PT vs GCDCA alone.

Anti-apoptotic effect of pertussis toxin is not dependent on the activation of ERK1/2, p38 MAP Kinase, PI3-kinase and protein kinase C

To investigate whether specific protein kinase pathways are involved in the anti-apoptotic effects of PT, GCDCA-exposed rat hepatocytes were co-treated with inhibitors of MAPK, PI3K and PKC. These inhibitors alone do not induce caspase-3 activity in rat hepatocytes (data not shown) [16]. The protective effect of PT against GCDCA-induced apoptosis was not abolished by inhibition of ERK1/2, p38 MAP kinases, PI3 kinase and PKC pathways (Figure3). This suggests that the anti-apoptotic effect of PT is independent of the activation of these cell survival signaling kinases or that parallel pathways contribute to the protective effect and that inhibition of only one pathway does not result in increased apoptotic cell death.

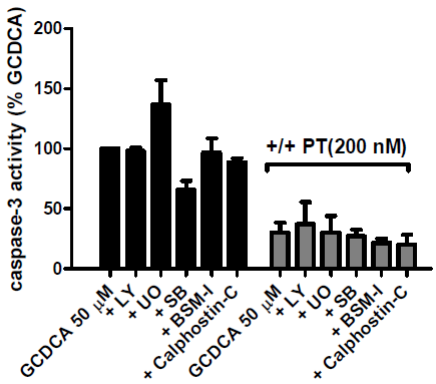

Figure3: The protective effect of pertussis toxin (PT) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis is not dependent on the activation of p38 MAP kinase, ERK1/2 MAPK, PI3K pathway and PKC pathway. Caspase-3 activity in rat hepatocytes treated with 50 µmol/L of GCDCA in the presence and absence of 200 nmol/L PT and with or without the inhibitors of ERK1/2- MAPK (10 µmol/L of U0126; U0), p38 MAPK (10 µmol/L of SB 203580; SB), PI3K (50 µmol/L of LY 294002; LY), PKC inhibitors (1 µmol/L of calphostin-C, 1 µmol/L of BSM-I).

Pertussis toxin protects hepatocytes against cytokine-induced caspase-3 activation and apoptotic nuclear morphology

We also analyzed the effect of PT on cytokine-induced apoptosis in hepatocytes. TNFα, in combination with ActD, induces caspase-3 activation in hepatocytes that peaks around 16 hours [21]. PT significantly reduced TNFα/ActD-induced caspase-3 activity in rat hepatocytes (-60%, P<0.05; Figure4A).

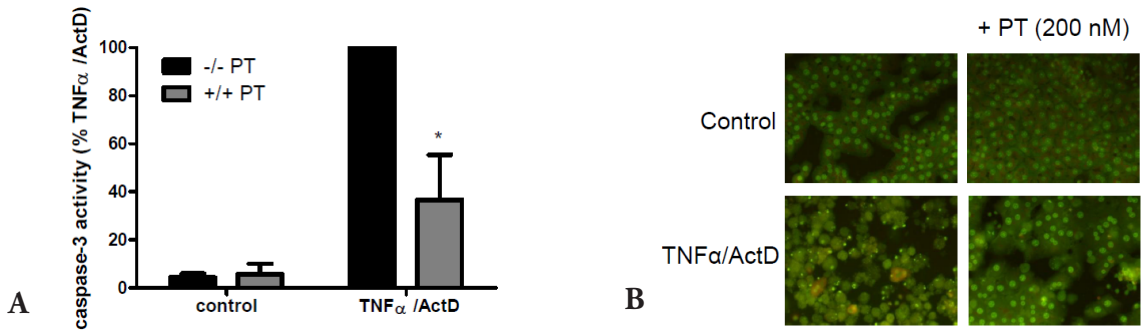

Figure4: Pertussis toxin (PT) inhibits tumor necrosis factor-α/actinomycin-D (TNFα/ActD)-induced caspase-3 activity and nuclear fragmentation. (A) Primary rat hepatocytes were treated for 16 hours with 20 ng/ml of TNFα in the presence of 200 ng/ml of ActD. 200 nmol/L of PT was added 30 min prior to the addition of TNFα/ActD. * P < 0.05 for TNFα/ActD + PT vs. TNFα/ActD alone. **(B)** Acridine orange staining. Treatment with TNFα/ActD induces nuclear condensation and fragmentation which is blocked with 200 nmol/L PT.

Acridine orange staining confirmed that TNFα/ActD-induced activation of caspase-3 resulted in the formation of fragmented and condensed nuclei, markers of end-stage apoptosis, after 16 hours (Figure4B). Importantly, theses markers were absent when TNFα/ActD-exposed hepatocytes were co-treated with PT, confirming that apoptosis was strongly inhibited in the presence of PT (Figure4B). Our data show that the PT-sensitive α-subunit of G-proteins is also a key player in TNFα-induced apoptotic signal transduction in primary rat hepatocytes.

Pertussis toxin does not inhibit apoptosis in HepG2-rNtcp cells

To investigate whether PT has the same anti-apoptotic effect in a human hepatocellular carcinoma cell line as it has in primary rat hepatocytes, we investigated the effect of PT in HepG2-rNtcp cells. HepG2-rNtcp cells were pre-treated with PT for 30 minutes, followed by exposure to GCDCA for 4 hours or TNFα/ActD for 16 hours. PT did not induce caspase-3 activity in HepG2-rNtcp nor did it inhibit GCDCA-induced caspase-3 activation in these cells (Figure5A). Similarly, PT did not reduce the TNFα/ActD-induced caspase-3 activity in these cells (Figure5B). These data suggest that the PT-sensitive Gα-protein is specifically involved in the apoptotic signaling pathways in primary hepatocytes and not in hepatocellular carcinoma cells.

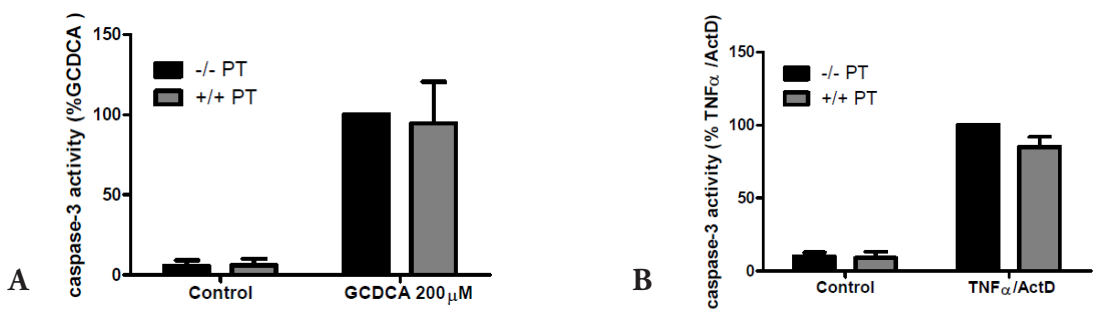

Figure5: Pertussis toxin (PT) does not inhibit apoptosis in HepG2-rNtcp cells. (A) Caspase-3 activity in HepG2-rNtcp cells treated with 200 μmol/L of GCDCA in the presence and absence of 200 nmol/L PT. **(B)** HepG2-rNtcp cells were treated for 16 hours with 20 ng/ml of TNFα in the presence of 200 ng/ml of ActD. 200 nmol/L of PT was added 30 min prior to the addition of TNFα/ActD.

Discussion

In this study, we report that PT, an inhibitor of Gα-proteins, protects primary rat hepatocytes against bile acid- and cytokine-induced apoptosis. These effects are specific for primary rat hepatocytes and are not observed in the human hepatocellular carcinoma cell line HepG2-rNtcp.

We demonstrate that the protective effect of PT is rapidly induced in hepatocytes and is sustained in rat hepatocytes, although the protective action of PT appears to be independent of a single protein kinase (-signaling pathway). We propose that the PT-sensitive α-subunit of G-proteins is a key player in apoptotic signal transduction in rat hepatocytes.

Among the PT-sensitive G-proteins, the Gi family is the largest family with wide expression in the different cells and Gβγ signaling is usually associated with the Gi family [8, 22]. Other PT-sensitive G-proteins have a more restricted distribution in neuroendocrine, visual and lingual tissues [8]. Regulation of gluconeogenesis by bile acids and regulation of lipoapoptosis by lysophosphatidilcholine has previously been shown to be mediated via GαiPCR-dependent mechanisms in hepatocytes [12, 14]. Our data suggest that PT-sensitive GPCRs (likely GαiPCRs) are involved in bile acid- and cytokine-induced apoptotic signal transduction in primary rat hepatocytes as well. Although the involvement of GαiPCRs in lipoapoptosis has been reported before [14], our data suggest that lipid mediators are not the only death effectors that use GαiPCRs dependent signaling pathways in hepatocytes.

The specific effect of PT (i.e. catalyzing ribosylation) on the α-subunit of Gi-proteins can explain the rapidly induced and sustained (protective) effect of PT in our experiments. ADP-ribosylated Gαi-proteins are in an "off "state and cannot transduce GPCR-induced signals [11, 23]. Therefore the receptors that are coupled to these G-proteins are not able to induce signaling pathways upon activation by agonists.

MAP kinase-, PI3 kinase- and PKC-signaling pathways play important roles in regulating hepatocyte death and survival in response to stress inducers [24-27]. In addition, cross-talk between GPCRs and these signaling pathways is responsible for cellular responses, such as cell proliferation, survival, migration and differentiation [28]. Interestingly, our findings indicate that the protective effect of the pertussis toxin against GCDCA-induced apoptosis is not grossly dependent on one of these signaling pathways and may actually be completely independent of these signaling cascades. This finding is remarkable because it has been reported that conjugated bile acids such as GCDCA induce phosphorylation of ERK1/2 and PI3K/Akt pathways in a PT-sensitive manner in primary rat hepatocytes [13]. Thus, one may assume that co-treatment with PT hypersensitizes hepatocytes to GCDCA-induced apoptosis via inhibiting cell survival pathways (e.g., ERK and PI3K/Akt). However, our findings demonstrate the opposite. It is accepted that PT is a general inhibitor for Gαi-proteins and inhibits several GαiPCRs signal transduction pathways [8]. Therefore, it is likely that despite the inhibitory effect of PT on GCDCA-induced ERK1/2 and PI3K/Akt phosphorylation, the overall effect of PT in GCDCA-treated hepatocytes favors cell survival.

TNFα/ActD induces apoptosis in hepatocytes via the activation of death receptor signaling cascade (extrinsic pathway), whereas GCDCA induces apoptosis in hepatocytes via the activation of the mitochondria-dependent apoptotic cascade (intrinsic pathway) [21, 29]. However, there is cross-talk between the bile acid-induced and cytokine-induced signaling cascades in hepatocytes [30, 31]. Ligand-independent transactivation of tyrosine kinase receptors, such as EGFR, is one of the crucial steps in the cross-talk between the intrinsic and the extrinsic apoptotic pathways in rat hepatocytes [31]. Tyrosine kinase receptor transactivation has been associated with GPCRs (reviewed in [32]). Given the involvement of EGFR transactivation in rat hepatocyte apoptosis and the anti-apoptotic effect of PT in rat hepatocytes (our data), we suggest that there is cross-talk between PT-sensitive GPCR/Gαi and the EGFR, which leads to apoptosis. Indeed, it has been observed that PT pretreatment of hepatocytes inhibited taurodeoxycholic acid (TDCA)-induced activation of the EGFR [13]. It is also reported that PT-sensitive Gi proteins are uniquely involved in the signal transduction pathway mediating EGF-induced activation of phospholipase C-gamma (PLCγ) and Ca^{2+} mobilization (via tyrosine phosphorylation of EGFR) in rat hepatocytes, but not in a rat liver cell line (WB) [33]. Whether GCDCA-induced activation of EGFR in primary hepatocytes is also PT-sensitive remains to be determined.

PT has no effect on apoptotic signaling pathways induced by GCDCA and TNFa/ActD in HepG2-rNtcp cells. This could be related to differences between (transformed) HepG2 cells and primary hepatocytes with regard to gene expression patterns, signaling cascades, GPCRs expression patterns and/or the ability of Gαi proteins to interact with the effector receptors such as EGFR [34, 35]. Indeed, it has been shown that Gαi proteins are unable to produce a stable complex with the EGFR or other EGFR-induced signaling protein kinases (such as PLCγ) in the rat epithelial liver cell line, WB [33].

It is known that liver injury is often accompanied by apoptotic hepatocyte cell death [1]. High levels of bile acids, cytokines, reactive oxygen species, drugs and toxins can induce hepatocyte apoptosis. In the liver, massive hepatocyte apoptosis results in acute liver failure, whereas persistent hepatocyte apoptosis and necrosis is often associated with activation of hepatic stellate cells and fibrogenesis, chronic liver dysfunction and end-stage liver disease [1, 3]. Ideally, the most direct therapeutic strategy is to eliminate the cause of the liver injury. However, effective treatments do not exist for many liver diseases, including primary sclerosing cholangitis, NASH, alcohol-mediated hepatitis, viral hepatitis unresponsive to antiviral therapies and acute liver failure. Anti-apoptotic interventions are among the most promising therapeutic strategies for these diseases as they may reduce the apoptosis induced-inflammation and fibrogenesis [1, 36, 37]. GPCRs, are suggested as

novel targets for drug innovation, as they have pivotal roles in many physiologic functions (e.g., cell proliferation, angiogenesis, survival) and in multiple diseases including the development of cancer and cancer metastasis. Excellent therapeutic benefits have been observed with some GPCR-based drugs in clinical trials: e.g., the endothelin A receptor antagonists ZD4054 and atrasentan show good antitumor efficacy for ovarian and prostate cancer [5, 6, 38, 39]. GPCRs (e.g., orexin receptor, GPCR78 and LPA receptor) are also involved in the regulation of apoptosis in cancer cells via interaction with different intracellular regulators of apoptosis such as MAPK-, NF-κB- and p53-associated pathways (reviewed in [6]). The participation of GPCR/Gαi in hepatocyte apoptosis suggests new targets for drug innovation for (chronic) liver diseases and liver cancer treatment in the future. E.g., activating death receptor-mediated apoptosis in cancer cells by death ligands (such as TRAIL, FasL and TNFα) is suggested as an effective therapeutic strategy in the treatment of hepatocellular carcinoma [40]; however, this strategy may lead to the induction of apoptosis in adjacent normal hepatocytes and loss of functional tissue. In this situation, an adjuvant anti-apoptotic therapy that will inhibit cell death in normal hepatocytes but has no effect on cancer cells may prevent excessive liver damage. Our data suggest that GPCR/Gαi-based therapeutic strategies may serve as the anti-apoptotic adjuvant therapy, protecting normal tissue against inflammation, bile acid and/or drug-induced cell death.

In summary, our data indicate that GPCRs/Gαi participates in several apoptotic signaling pathways in hepatocytes, but not in human hepatocellular carcinoma cells, and that inhibiting the αi subunit of G-proteins is a very effective anti-apoptotic strategy in vitro. The participation of GPCR/Gαi in hepatocyte apoptosis unveils new targets for drug innovation to treat liver diseases in the future.

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Chapter 6

Sphingosine kinase-1 inhibition protects primary rat hepatocytes against bile acid-induced apoptosis

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In preparation

Abstract

Background and Aim: Sphingosine kinases (SphKs) and their product sphingosine-1-phosphate (S1P) have been reported to regulate apoptosis and survival of liver cells. Cholestatic liver diseases are characterized by cytotoxic levels of bile salts inducing liver injury. It is unknown whether SphKs and/or S1P play a role in this pathogenic process. Here, we investigated the putative involvement of SphK1 and S1P in bile salt-induced cell death in hepatocytes.

Methods: Primary rat hepatocytes were exposed to glycochenodeoxycholic acid (GCDCA) to induce apoptosis. GCDCA-exposed hepatocytes were co-treated with S1P, the SphK1 inhibitor Ski-II and/or specific antagonists of S1P receptors (S1PR1 and S1PR2). Apoptosis (by caspase-3 assay and Acridine orange staining) and necrosis (by Sytox green staining) were quantified.

Results: Ski-II significantly reduced GCDCA-induced apoptosis in hepatocytes (-70%, $p < 0.05$) without inducing necrosis. GCDCA induced Ca2+ oscillations in hepatocytes and co-treatment with the Ca2+ chelator BAPTA repressed GCDCA-induced apoptosis. Ski-II inhibited the GCDCA-induced intracellular [Ca2+] oscillations. Transcripts of all five S1P receptors were detected in hepatocytes, of which S1PR1 and S1PR2 appear most dominant. Inhibition of S1PR1, but not S1PR2, reduced GCDCA-induced apoptosis by 20%. Exogenous S1P also significantly reduced GCDCA-induced apoptosis (-50%, $p < 0.05$), however, in contrast to the GCDCA-induced (intracellular) SphK1 pathway, this was dependent on S1PR2 and not S1PR1.

Conclusion: Our results indicate that SphK1 plays a pivotal role in mediating bile salt-induced apoptosis in hepatocytes in part by interfering with intracellular [Ca2+] signaling and activation of S1PR1. Thus, inhibition of SphK1 may be an attractive therapeutic strategy to prevent hepatocyte apoptosis in patients with cholestatic liver disease.

Introduction

Sphingosine kinases (SphK1 and SphK2) are a distinct class of lipid kinases that are rapidly activated by a variety of extracellular and/or intracellular stimuli and are implicated in modulating cell survival and apoptosis [1]. Additionally, activation of SphKs results in activation of inflammatory processes, including generation of inflammatory cytokines and reactive oxygen species [2-4]. SphKs catalyze phosphorylation of sphingosine to generate sphingosine 1-phosphate (S1P) [5, 6]. Interestingly, S1P has been reported to trigger both survival and apoptotic signals in different cell types [5, 7-13]. S1P acts as a first messenger through the G protein coupled receptors (GPCRs), termed $S1PR_1$, $S1PR_2$, $S1PR_3$, $S1PR_4$ and $S1PR_5$, which are differentially expressed in different tissues. The cell type-specific expression of S1P receptors (S1PRs), as well as their differential coupling to different G-proteins, provides a clue to the different functions of S1P [13]. Additionally, S1P can act as a second messenger in various cell types by activating calcium channels and increasing intracellular [Ca^{2+}], thereby regulating cell proliferation, growth, migration and apoptosis [13, 14].

In chronic (cholestatic) liver diseases, the liver is exposed to increased levels of bile salts, cytokines and reactive oxygen species, all of which independently can lead to loss of functional liver mass due to hepatocyte cell death. Concomitantly, hepatic stellate cells become activated, start proliferating and produce excessive amounts of extracellular matrix proteins leading to liver fibrosis, which may progress to end-stage liver disease [15]. Hence, identifying the mechanisms of hepatocyte injury is crucial for developing new therapeutic strategies to prevent hepatocyte apoptosis. We have previously shown that the sodium-taurocholate cotransporting polypeptide (NTCP) is crucial for conjugated bile salt-induced hepatocyte apoptosis [16], suggesting that bile salt-induced intracellular signaling pathways play important roles in modulating hepatocyte apoptosis. Additionally, it has been shown that bile salt-induced apoptosis is a mitochondria-dependent process which coincides with increased intracellular [Ca^{2+}] levels [17]. Interestingly, it has been suggested that SphK2 inhibition improves mitochondrial function and hepatocyte survival after hepatic ischemia-reperfusion [18]. However, the role played by SphK1 in hepatocyte apoptosis is currently unclear. In the present study we have investigated the role played by SphK1 in two models of bile salt- and cytokine-induced apoptosis in rat hepatocytes. We demonstrate that SphK1 plays a pivotal role in mediating bile salt-induced apoptosis in hepatocytes, but this kinase has no effect in cytokine-induced apoptosis. Additionally, we investigated the effect of SphK1 inhibition on bile salt-induced intracellular [Ca^{2+}] mobilization.

Materials and Methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Charles River Laboratories Inc (Wilmington, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

Experimental design

Hepatocytes were isolated and cultured in mutiwell plates (Greiner bio-one) in William's E medium in a humidified incubator at 37 ºC and 5% CO2 as described before [16]. Experiments were started after the attachment period of 4 hours. In order to induce apoptosis, monolayers of cultured primary hepatocytes were exposed to 50 μM GCDCA (Sigma-Aldrich, St Louis, MO, USA) for 4 hours or 20 ng/ml recombinant murine TNFα (R&D Systems, Abingdon, U K) for 16 hours. Where indicated, hepatocytes were treated with 10 μmol/L S1P (Sigma-Aldrich) prior to the exposure to the apoptotic stimulus. The following specific inhibitors were used: Ski II (Tocris Biosciences, Bristol, UK) inhibitor of SphK1 (1-10 μM), VPC23019 (Tocris Biosciences) antagonist of $S1PR_1$ (5 or 10 μM), JTE-013 (Tocris Biosciences) antagonist of $S1PR_2$ (5 or 10 μM), U0126 (Promega, Madison, USA) inhibitor of ERK1/2 (10 μM), SB 203580 (VWR, Amsterdam, the Netherlands) inhibitor of p38 MAPK (10μM), LY 294002 (VWR) inhibitor of PI3 kinase (50 μM), Calphostin-C and Bisindolylmaleimide I (BSM-I) (VWR) inhibitors of protein kinase-C (1 μM), actinomycin-D (Roche Diagnostics, Almere, the Netherlands) transcriptional inhibitor (200 ng/ml). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 minutes prior to the apoptotic stimuli unless stated otherwise. The intracellular [Ca^{2+}] chelator, BAPTA (25 μM, Sigma-Aldrich), was added 30 minutes prior to GCDCA. Hepatocytes cultured in HBSS (containing [Ca^{2+}]/ [Mg^{2+}]) instead of William's E medium were used as an internal control in these experiments. All experiments were performed in triplicate and each experiment was repeated at least three times using hepatocytes from different rats. Cells were harvested at the indicated time points as described previously [16].

Apoptosis assay

Caspase-3 activity was measured as described previously [16]. The arbitrary fluorescence unit (AFU) was corrected for the amount of protein. Protein concentration was determined using a commercially available kit (Bio-Rad, Veenendaal, the Netherlands).

Quantitative PCR

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR) were performed as described previously [19] and mRNA expression levels were calculated relative to the housekeeping gene 18S. Primers and probes are listed in Table 1.

Determination of intracellular [Ca^{2+}] changes in hepatocytes

Freshly isolated hepatocytes were loaded with the Ca^{2+}-probe Fura2 (3 μM, Molecular Probes, US) and Ca^{2+} imaging experiments were performed one hour after plating as described previously [20].

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± SD. Two way ANOVA was used to determine significance of differences between experimental groups. A p-value of less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

Sphingosine kinase-1 inhibition protects hepatocytes against GCDCA-induced apoptosis, but has no effect on TNFα/ActD-induced apoptosis

GCDCA (at 50 μM) and TNFα/ActD induce caspase-3 activity in primary rat hepatocytes

that peaks after 4 h- and 16 h-exposure, respectively [16]. Therefore, the effect of Ski II, a selective inhibitor of SphK1 [21], on GCDCA- and TNFα/ActD-induced caspase-3 activity was investigated at these time points. Ski II significantly inhibited the GCDCA-induced caspase-3 activity up to 70% ($p<0.05$), in a concentration-dependent manner (Figure1A). Exposure of primary rat hepatocytes to Ski II (1-10 μM) alone did not induce caspase-3 activity, nor did it reduce the caspase-3 activity in TNFα/ActD-treated rat hepatocytes (Figure1A and B). Since no toxic effects of Ski II were observed at the highest concentration used, a concentration of 10 μM was used for all subsequent experiments. These data show that inhibition of SphK1 specifically protects primary rat hepatocytes against GCDCA-induced apoptosis indicating that SphK1 activity is pro-apoptotic in these cells.

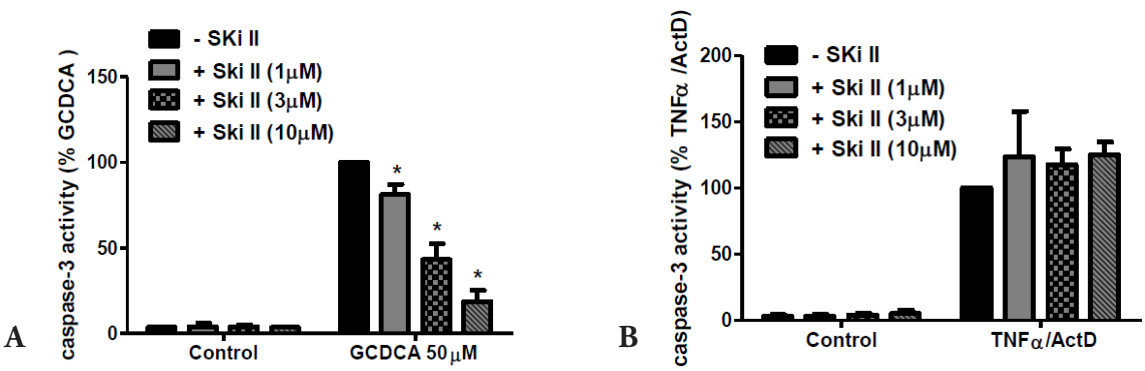

Figure1: Sphingosine kinase-1 (SphK1) inhibitor (Ski II) inhibits glycochenodeoxycholic acid (GCDCA)-induced apoptosis in rat hepatocytes but does not inhibit tumor necrosis factor α/Actinomycin-D (TNFα/ActD)-induced apoptosis. (A) Primary rat hepatocytes were treated for 4 hours with 50 μM of GCDCA, 1-10 μMof Ski II or a combination of both. Ski II was added 30 min prior to the addition of GCDCA. * $P < 0.05$ for GCDCA + Ski II vs GCDCA alone. **(B)** Hepatocytes were treated for 16 hours with TNFα (20 ng/ml)/ActD (200 ng/ml), 1-10 μM of Ski II or a combination of both. Ski II was added 30 min prior to the addition of TNFα/ActD.

The anti-apoptotic effect of SphK1 inhibition is independent of the activation of ERK1/2, p38 MAP Kinase, PI3-kinase, protein kinase C and gene transcription

To investigate whether specific kinase pathways are involved in the anti-apoptotic effects of Ski II, GCDCA-exposed rat hepatocytes were co-treated with inhibitors of MAPK, PI3K or PKC. These inhibitors alone did not induce caspase-3 activity in rat hepatocytes (data not shown) nor did they induce caspase-3 activity in hepatocytes co-treated with Ski II (Figure2). Importantly, the protective effect of Ski II against GCDCA-induced caspase-3 activation was not abolished by inhibition of ERK1/2, p38 MAPK, PI3K and PKC (Figure2), suggesting that the anti-apoptotic effect of Ski II is essentially independent of the activation of these kinase pathways. These inhibitors

at the concentrations tested here were effective in our positive control experiments (data not shown). In addition, we studied the role of gene transcription in the protective effect of Ski II against GCDCA-induced apoptosis. Actinomycin D (200 ng/ml) is an inhibitor of gene transcription and sensitizes hepatocytes to TNFα-induced apoptosis, but has no effect on GCDCA-induced caspase-3 activity in rat hepatocytes ([16] and our data). Also, ActD treatment did not abolish the protective effect of Ski II against GCDCA-induced caspase-3 activation (Fig. 2). This finding suggests that adaptive gene regulation is not required for the anti-apoptotic effect of Ski II against GCDCA-induced apoptosis.

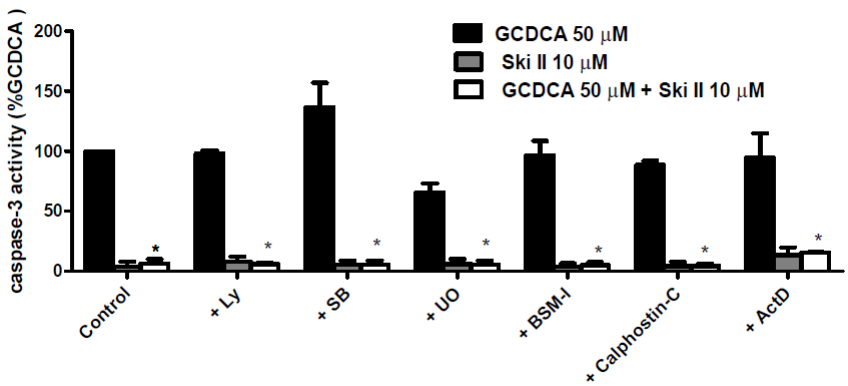

Figure2: The protective effect of sphingosine kinase-1 inhibitor (Ski II) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis is not dependent on the activation of p38 MAP kinase, ERK1/2 MAPK, PI3K pathway, PKC pathway and gene transcription. Caspase-3 activity in rat hepatocytes treated with 50 μM of GCDCA in the presence and absence of Ski II (10 μM) and with or without the inhibitors of ERK1/2- MAPK (10 μM of U0126; U0), p38 MAPK (10 μM of SB 203580; SB), PI3K (50 μM of LY 294002; LY), PKC inhibitors (1 μM of calphostin-C and BSM-I), transcription inhibitor (200 ng/ml of ActD). * $P < 0.05$ for GCDCA+ Ski II vs. GCDCA. The differences between gray and white bars are not significant.

GCDCA-induced apoptosis is dependent on mobilization of intracellular [Ca^{2+}] and SphK1 inhibition blunts GCDCA-induced [Ca^{2+}] oscillations in rat hepatocytes

It was previously shown that GCDCA-induced apoptosis coincides with intracellular [Ca^{2+}] mobilization from the ER, caspase-12 activation and mitochondrial dysfunction resulting in leakage of cytochrome c [17], suggesting that intracellular [Ca^{2+}] mobilization contributes to GCDCA-induced apoptosis. We investigated the effect of BAPTA (25 μM), a chelator of intracellular [Ca^{2+}] , on apoptosis in GCDCA-exposed hepatocytes. BAPTA did not increase caspase-3 activity in hepatocytes when added alone (Figure3A). Importantly, the GCDCA-induced caspase-3 activity was significantly reduced in BAPTA loaded hepatocytes (-50%, $P<0.05$), confirming that the

mobilization of intracellular [Ca^{2+}] pools plays an important role in GCDCA-induced apoptotic signaling. The same results was obtained when hepatocytes cultured in HBSS (containing [Ca^{2+}] / [Mg^{2+}]), serving as controls for experiments performed in Williams' E medium, were analyzed (Figure3A). We investigated the pattern of intracellular [Ca^{2+}] mobilization in GCDCA-exposed fura2-loaded hepatocytes and the effect of SphK1 inhibition on these intracellular [Ca^{2+}] mobilization.

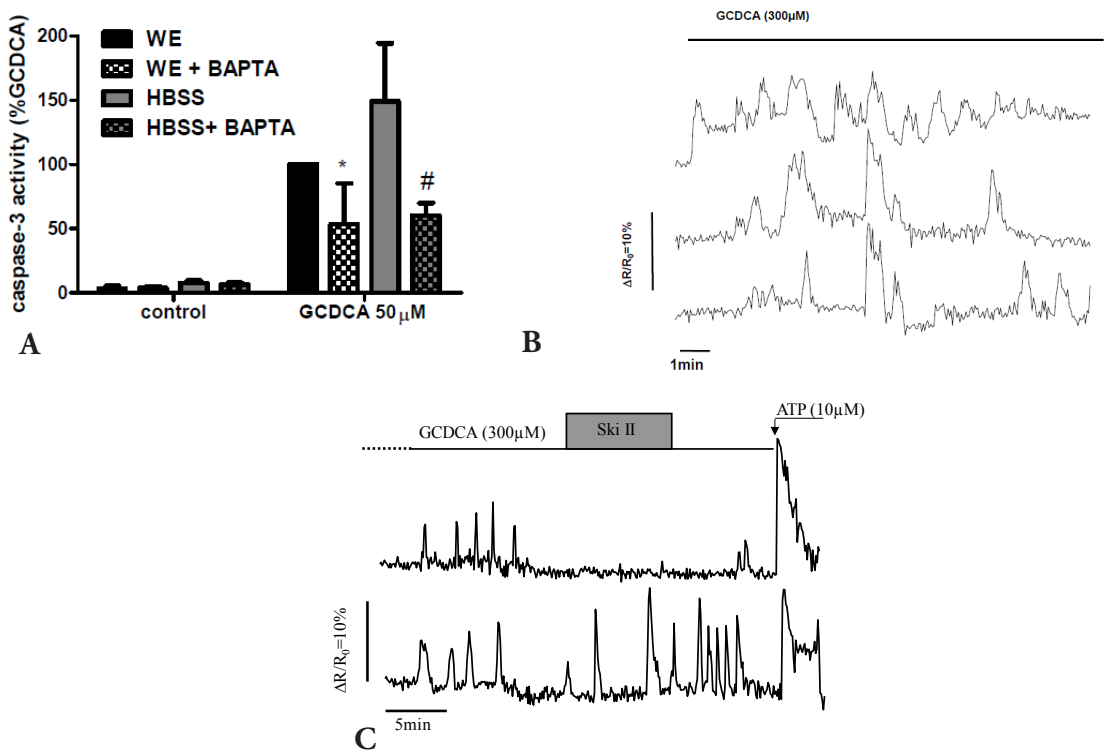

Figure3: Glycochenodeoxycholic acid (GCDCA)-induced apoptosis in rat hepatocytes is dependent on the intracellular [Ca^{2+}] mobilizations and sphingosine kinase-1 (SphK1) inhibition blunts GCDCA-induced [Ca^{2+}] oscillations in rat hepatocytes. (A) Primary rat hepatocytes were plated in the William's E medium (WE) or Hank's buffered salt solution (HBSS) containing calcium and magnesium. Caspase-3 activity in rat hepatocytes treated with GCDCA (50μM) and with or without BAPTA (25 μM). Hepatocytes were loaded with BAPTA 30 minutes prior to the addition of GCDCA. * $P < 0.05$ for GCDCA+ BAPTA vs. GCDCA (in WE) and # $P < 0.05$ for GCDCA+ BAPTA vs. GCDCA (in HBSS). **(B)** The figure shows measurements of intracellular [Ca^{2+}] oscillations in three representative hepatocytes loaded with Fura2-AM. Cells were incubated with 300 μM GCDCA and [Ca^{2+}] oscillations were measured during 15-20 minutes. The traces shown are representative of those obtained in at least three independent experiments. **(C)** The figure shows measurements of intracellular [Ca^{2+}] oscillation in two representative hepatocyte loaded with Fura2-AM. Cells were first perfused with 300 μM GCDCA (the left part of the graph in continuous line) and then with GCDCA (300 μM) + Ski II (10 μM), successively (the grey box). Subsequently cells were re-perfused with GCDCA (the right part of the graph). The traces shown are representative of those obtained in at least three independent experiments. [Ca^{2+}]i oscillations were inhibited or less frequent in the presence of Ski II in 65% of hepatocytes that were individually imaged. ATP (10 μM) was used as an internal control.

Our data showed that GCDCA (at 300 μM) in the presence of extracellular [Ca^{2+}], induces

[Ca^{2+}]oscillations in rat hepatocytes starting about 2-8 minutes after the addition of GCDCA (Fig. 3B). Incubation with 300 μM GCDCA did not induce necrosis in hepatocytes up to at least one hour (data not shown).We did not observe [Ca^{2+}] oscillations with concentration below 300 μM GCDCA, likely due to the experimental limitations. Interestingly, the [Ca^{2+}] oscillations induced by GCDCA were not coordinated in hepatocytes nor were they homogenously induced in all cells at the same time (Figure3B) unlike [Ca^{2+}] oscillations induced by angiotensin II (see below). Importantly, these GCDCA-induced [Ca^{2+}] oscillations were blunted in the presence of Ski II (10μM) in 65% of hepatocytes, which showed [Ca^{2+}] oscillations induced by GCDCA (Figure3C), suggesting that GCDCA-induced [Ca^{2+}] oscillations in these hepatocytes are mediated via S1P produced by SphK1. When added alone Ski II did not induce calcium oscillations (data not shown).

$S1PR_1$-dependent signaling contributes to the pro-apoptotic effect of GCDCA while exogenous S1P protects hepatocytes against GCDCA-induced apoptosis via interaction with $S1PR_2$

S1P acts as a first messenger via its receptors, $S1PR_{1-5}$ [13]. We detected significant mRNA expression levels of all S1P receptors in rat hepatocytes (Figure4A). $S1PR_1$ and $S1PR_2$ were expressed more abundantly compared to the others, which is in accordance with a previous report [22]. We investigated the effect of inhibiting $S1PR_1$ and S1PR2 on GCDCA-induced apoptosis using specific antagonists of $S1PR_1$ (VPC23019, at 5 and 10μM) and $S1PR_2$ (JTE-013, at 5 and 10μM), respectively. None of these S1P receptor antagonists induced caspase-3 activation when added to hepatocytes alone (Figure4B). The apoptotic effect of GCDCA was reduced significantly (-20%, $P<0.05$) in the presence of $S1PR_1$ antagonist, indicating that $S1PR_1$-dependent signaling contributes to the pro-apoptotic effect of GCDCA (Figure4B). In contrast, the $S1PR_2$ antagonist did not significantly inhibit GCDCA-induced caspase-3 activity, suggesting that GCDCA-induced apoptosis is not mediated via $S1PR_2$-dependent signaling. In fact, hepatocytes may become even more sensitive to the toxic effect of GCDCA in the presence of the $S1PR_2$ antagonist (Figure4B).

Endogenous S1P can induce intracellular signaling pathways independent of S1P receptors and/or via interaction with S1P receptors while exogenous S1P interacts with S1P receptors. Therefore, different functions can be expected from endogenous S1P and exogenous S1P [8, 13, 23]. Consequently, we also investigated the effect of exogenous S1P (10 μM) on GCDCA-induced apoptosis in hepatocytes. The concentration of exogenous S1P (100 nM- 10 μM) that causes various responses in cells is comparable with the plasma and tissue concentrations of

S1P in vivo [23-26]. When added alone, S1P (10 μM) did not induce caspase-3 activation in rat hepatocytes, but significantly inhibited GCDCA-induced caspase-3 activity (-50%, P<0.05; Figure4C). Interestingly, the protective effect of exogenous S1P against GCDCA-induced apoptosis was reversed in hepatocytes co-treated with the S1PR2 antagonist (JTE-013, 10μM), but not in hepatocytes co-treated with the $S1PR_1$ antagonist (VPC23019, 10μM; Figure4C).

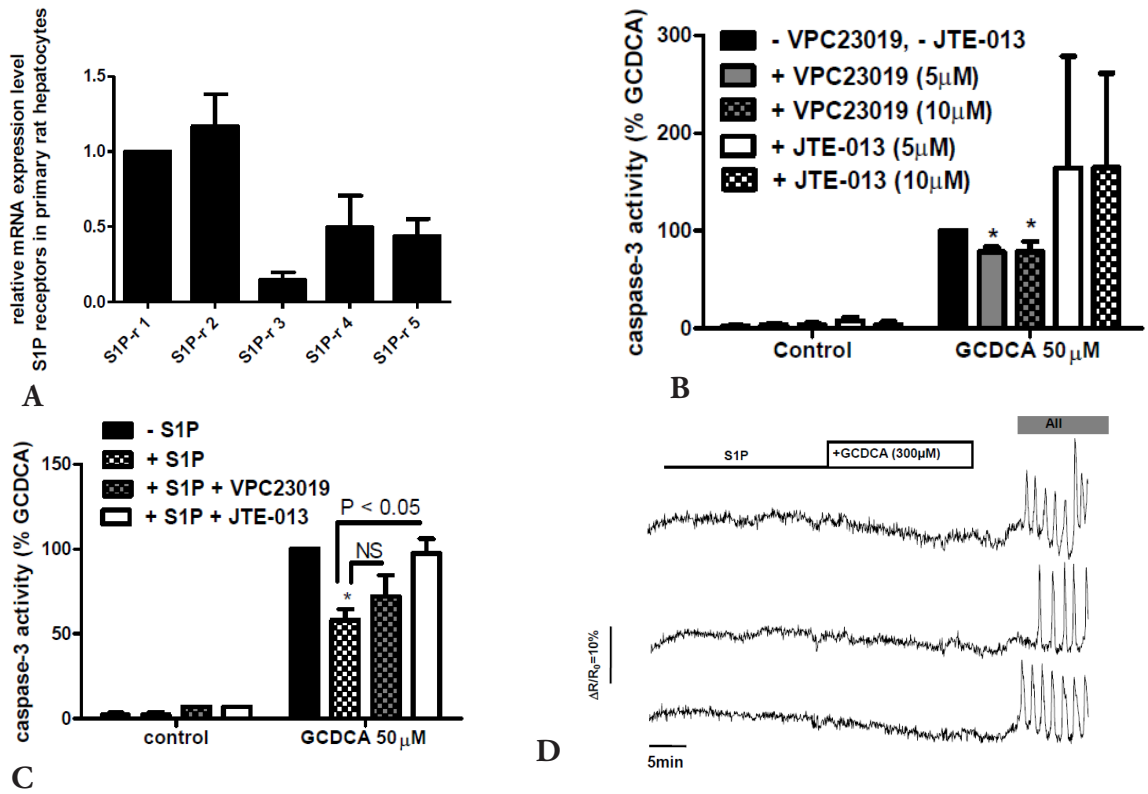

Figure4: Sphingosine-1 phosphate receptor 1 ($S1PR_1$)-dependent signaling contributes to pro-apoptotic effect of GCDCA but not Sphingosine-1 phosphate receptor 2 ($S1PR_2$), while exogenous sphingosine-1 phosphate (S1P) protects hepatocytes against GCDCA-induced apoptosis via interaction with $S1PR_2$. (A) Expression pattern of S1P receptors (S1P-r 1-5) in rat hepatocytes. qPCR analysis for S1P receptors using cDNA of rat hepatocytes in primary cultures at 4 hours after plating. **(B)** Hepatocytes were treated with 5 and 10 μM of $S1PR_1$ antagonist (VPC23019) or $S1PR_2$ antagonist (JTE-013) 30 minutes prior to the addition of GCDCA (50 μM). * P < 0.05 for GCDCA+ VPC23019 vs. GCDCA. **(C)** Primary rat hepatocytes were treated for 4 hours with 50 μM of GCDCA with or without 10 μM of S1P in the medium. $S1PR_2$ antagonist (JTE-013, 10μM) and $S1PR_1$ antagonist (VPC23019, 10μM) were added 30 min prior to the addition of S1P. * P < 0.05 for GCDCA+ S1P vs. GCDCA alone. **(D)** The upper figure shows measurements of intracellular [Ca2+] oscillation in three representative hepatocytes loaded with Fura2-AM. Cells were first incubated with 10 μM S1P and perfused with S1P (10 μM) + GCDCA (300 μM), successively. Subsequently cells were washed and perfused with angiotensin II (AII, 10 nM), serving as a positive control. The traces shown are representative of those obtained in at least three independent experiments using individual rat hepatocytes.

Exogenously added S1P (10 μM) did not induce $[Ca^{2+}]$ oscillations in rat hepatocytes (Figure4D)

and prevented . GCDCA-induced [Ca^{2+}] oscillations. S1P did not lead to irreversible blockade of [Ca^{2+}] oscillations as those could subsequently be induced by the positive control, angiotensin II (AII, 10 nM; Figure4D). These data indicate that exogenous S1P has anti-apoptotic effects in primary rat hepatocytes and acts via a $S1PR_2$-dependent signaling pathway.

Discussion

In the present study, we show that SphK1 inhibition reduces GCDCA-induced apoptosis in primary rat hepatocytes. We further demonstrate that the protective effect of Ski II occurs independently of various specific kinase pathways and of adaptive gene regulation. However, it is dependent on the mobilization of intracellular [Ca^{2+}] and Ski II inhibits GCDCA-induced [Ca^{2+}] oscillations. We suggest that $S1PR_1$-dependent signaling contributes to the pro-apoptotic effect of GCDCA. In contrast, exogenous S1P promotes cell survival against GCDCA-induced apoptosis via $S1PR_2$-dependent signaling in rat hepatocytes (Figure5).

SphKs and S1P have been implicated to play a role in apoptosis and survival of liver cells [1, 8, 18]. SphK2 activation leads to the mitochondrial dysfunction and hepatocyte apoptosis after hepatic ischemia-reperfusion injury [18]. S1P may activate both apoptotic and survival pathways at the same time and the balance between the two pathways determines the end result. For instance, S1P is pro-apoptotic in human hepatic myofibroblasts acting via a receptor-independent pathway, while concomitant signaling via S1P receptors is anti-apoptotic [8]. The effect of SphK1 activation and S1P on hepatocyte apoptosis has not yet been elucidated. Hepatocyte apoptosis in response to elevated levels of toxic stimuli, such as bile salts, cytokines and reactive oxygen species, contributes to the pathogenesis of chronic liver diseases. Understanding the mechanisms of hepatocyte injury is therefore of great relevance for the development of therapeutic strategies to prevent hepatocyte apoptosis. Therefore, we studied the role played by SphK1 and S1P in in vitro models of bile salt (GCDCA)- and cytokine (TNFα)-induced hepatocyte apoptosis.

In our study, we have used Ski II to inhibit SphK1 signaling. Ski II is a selective inhibitor of SphK1 that does not act on the ATP-binding site of the enzyme and was shown to be highly specific for SphK1 up to 60 μM [21]. Using this strategy, our data indicate that GCDCA-induced apoptosis is in part dependent on SphK1 signaling in rat hepatocytes. In contrast to a previous report [10], we demonstrate that TNFα-induced apoptosis in rat hepatocytes is not SphK1-dependent. Osawa et al [10], analyzed the role of SphKs in human hepatoma cell lines and used non-selective (broad range)

inhibitors (DMS and DHS) of SphKs. In our study, we analyzed primary rat hepatocytes and focused on the role of SphK1 by applying a highly selective inhibitor (Ski II). These experimental differences may explain the fact why we did not observe an effect of inhibition of SphK1 on TNFα-induced apoptosis.

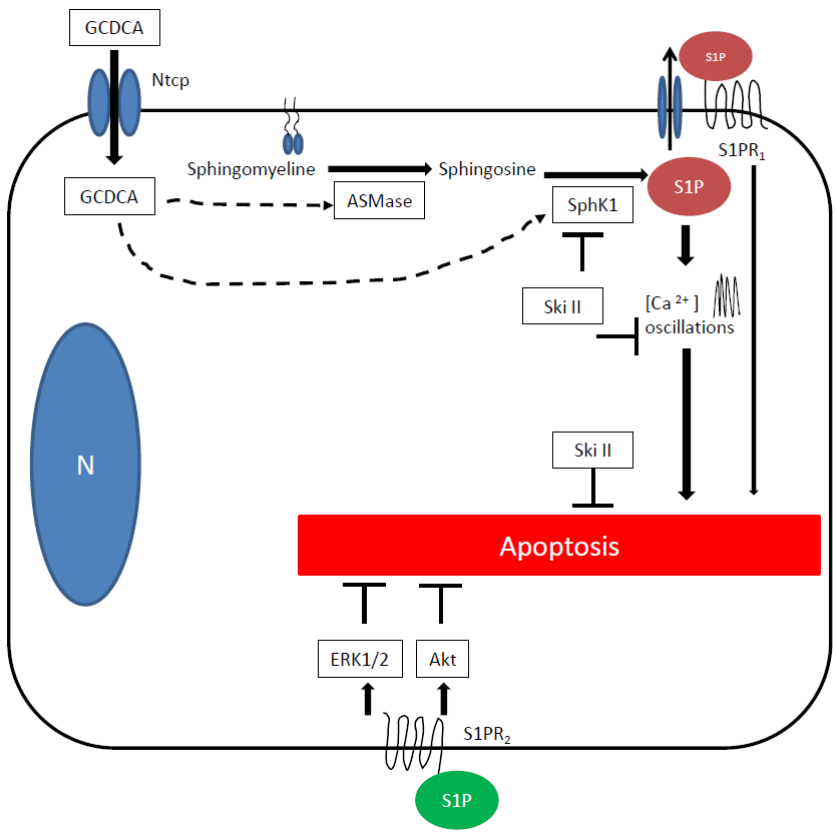

Figure5: Schematic drawing summerizing the proposed mechanisms for glychochenodeoxycholic acid (GCDCA)-induced apoptosis in rat heptocytes involving sphingosine kinase-1 (SphK1), sphingosine-1 phosphate (S1P) and sphingosine-1 phosphate receptors ($S1PR_1$ and $S1PR_2$). ASMase: acidic sphingomyelinase, Ntcp: sodium-taurocholate cotransporting polypeptide, Ski II: Sphk1 inhibitor.

Endogenous S1P was shown to function as a powerful mediator of $[Ca^{2+}]$ release through a non-InsP3 receptor-mediated mechanism in the ER membrane [27, 28]. Furthermore, it has been demonstrated that UTP stimulates $[Ca^{2+}]$ mobilization in a SphK-dependent manner, which is not mimicked by exogenous S1P [29]. Altogether, these reports suggest that endogenous S1P generated by SphKs activation induces intracellular $[Ca^{2+}]$ mobilization. GCDCA-induced apoptosis in rat hepatocytes has been shown to coincide with elevated intracellular $[Ca^{2+}]$ levels [17]. Accordingly, we show that GCDCA-induced apoptosis is dependent on intracellular calcium mobilization, since the GCDCA-induced apoptosis was reduced in hepatocytes loaded with the $[Ca^{2+}]$ chelator BAPTA. In our experiments, the GCDCA-induced increase in $[Ca^{2+}]$ is coinciding with $[Ca^{2+}]$

oscillations. Remarkably, not all hepatocytes showed [Ca^{2+}] oscillations in response to bile salts and the [Ca^{2+}] oscillations were not coordinated in hepatocytes. It was previously shown that the lack of coordination of [Ca^{2+}] oscillations in hepatocytes can result either from the absence of InsP3 production by bile salts or from the inhibitory effect of bile salts on the permeability of gap junction channels [30, 31]. Although we did not specifically test these mechanisms, they may provide a clue to explain the lack of homogeneity and coordination of [Ca^{2+}] oscillations induced by GCDCA in our experiments. Interestingly, we show that GCDCA induces [Ca^{2+}] oscillations in a SphK1-dependent manner which, importantly, is not mimicked by exogenous S1P at the concentration that is anti-apoptotic in hepatocytes. It has previously been shown that exogenous S1P mobilizes intracellular [Ca^{2+}] in rat hepatocytes [14, 32], but this did not present as [Ca^{2+}] oscillations. Therefore, we suggest that endogenous S1P generated by GCDCA-mediated pathways can act as a second messenger to induce [Ca^{2+}] oscillations in rat hepatocytes. Inhibition of SphK1 activation inhibits GCDCA-induced apoptosis as well as GCDCA-induced [Ca^{2+}]oscillations.

[Ca^{2+}]oscillations are implicated in the regulation of different cellular processes in hepatocytes including glucose metabolism and cell proliferation [33, 34]. [Ca^{2+}] oscillations are reported to regulate transcriptional activity in cells [35, 36] although our data indicate that neither GCDCA-induced apoptosis (18) nor the protective action of Ski II (present study) required adaptive gene regulation. Some cholestatic bile salts have been shown to increase intracellular [Ca^{2+}] and induce [Ca^{2+}] oscillations in rat hepatocytes [31, 37]. In the present study, we suggest that bile salt-induced [Ca^{2+}] oscillations in rat hepatocytes promote apoptosis, however, the exact pathway of the [Ca^{2+}] oscillation-induced events in regulating apoptosis remains unclear. It is reported that TUDCA (a choleretic anti-apoptotic bile salt [16]) can also induce [Ca^{2+}] oscillations in the H4-IIE cell line [38], however, this finding could not be confirmed in rat hepatocytes [31]. In addition, it is reported that TUDCA plays an important role in maintaining intracellular calcium homeostasis by reducing thapsigargin-induced release of [Ca^{2+}] from ER stores, resulting in the maintenance of mitochondrial membrane potential and cell survival [39]. It is therefore highly likely that cholestatic bile salt-induced [Ca^{2+}] oscillations in rat hepatocytes constitute a pro-apoptotic signal in these cells.

Activation of MAP-kinase signaling, PI3-Kinase signaling and PKC signaling pathways in hepatocytes play an important role in determining the balance between death and survival in response to stress [16, 40-43]. In addition, S1P and S1P receptors are reported to interact with these kinases [8, 10, 22, 44]. We hypothesized that the inhibition of these kinases would re-sensitize Ski II treated hepatocytes to GCDCA-induced apoptosis, but our data demonstrate that inhibition of ERK, PI3-kinase and/or PKC pathways are not involved in the protective effect of Ski II.

In the present study, we observe opposing functions for exogenous S1P versus endogenous S1P. Many reports indicate that S1P acts as a first messenger on cell surface S1P receptors in a paracrine and/or autocrine manner (reviewed in [45]). We suggest that in GCDCA-induced apoptosis, the main autocrine and/or paracrine role of S1P as a first messenger is to activate $S1PR_2$-dependent survival signaling. It has been shown that in rat hepatocytes exogenous S1P can activate ERK1/2 and Akt via interaction with $S1PR_2$ [22]. Additionally, activation of ERK1/2 and Akt signaling pathways contribute to hepatocyte survival [16]. Activation of theses pathways could contribute to the protective effect of exogenous S1P against GCDCA-induced apoptosis. Interestingly, the GCDCA-induced activation of $S1PR_1$-dependent signaling (likely by secreted endogenously generated S1P) is pro-apoptotic for rat hepatocytes, however, this pathway does not seem to play a major role in GCDCA-induced apoptosis as the antagonist of $S1PR_1$ reduced GCDCA-induced apoptosis only by 20%. Therefore, our evidence that GCDCA-induced apoptosis is a SphK1-dependent event indicates that the generated endogenous S1P acts as a second messenger to promote cell apoptosis in rat hepatocytes. Therefore, inhibiting SphK1 in hepatocytes could be an effective anti-apoptotic strategy in the treatment of cholestatic liver disease.

In summary, the results of this study demonstrate that GCDCA induces apoptosis in rat hepatocytes in a SphK1-dependent manner and suggest that endogenously generated S1P act as a second messenger to induce $[Ca^{2+}]$ oscillations and apoptosis in rat hepatocytes.

Table 1: Sequences of primers and probes used for quantitative PCR analysis

18 S rat	Sense Antisense Probe	5'-CGGCTACCACATCCAAGGA- 3' 5'-CCAATTACAGGGCCTCGAAA-3' 5'FAM-CGCGCAAATTACCCACTCCCGA- TAMRA3'
S1P-r 1 rat	Sense Antisense Probe	5'-GCGGACGCAGCTTCGTC- 3' 5'-CAGCAAGCAATCCGATGCTT- 3' 5'FAM-CTCCGAGAAACAGCAGCCTCGCTCA- TAMRA3'
S1P-r 2 rat	Sense Antisense Probe	5'-CGGCCTAGCCAGTGCTCA- 3' 5'-CGTCTCCTTGGTGTAATTGTAGTGTT- 3' 5'FAM-CCGGCCACTGAGCCCCACCAT- TAMRA3'
S1P-r 3 rat	Sense Antisense Probe	5'-GCATGCAACCAGCCCAGAT- 3' 5'-CGCCAGGAACGTTCATTTCAG- 3' 5'FAM-CTGCACGCCTTCAGGAACCCACC- TAMRA3'
S1P-r 4 rat	Sense Antisense Probe	5'-TGCTCTTTTGTGTGGTGGTCTT- 3' 5'-GTTGGCTCGGACCACCCTA- 3' 5'FAM-CTATCCTGAGCCTCTACGGGGCCATC- TAMRA3'
S1P-r 5 rat	Sense Antisense Probe	5'-TACGCCAAGGCCTATGTGCT- 3' 5'-CACCTGACAGTAAATCCTTGCATAGA- 3' 5'FAM-CCTTCCTGGGCATCCTGGCTGC- TAMRA3'

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Chapter 7

General Discussion

Hepatocyte apoptosis is ubiquitous in liver diseases [1-6]. Although apoptosis is primarily a non-inflammatory process responsible for the removal of excess or damaged cells, apoptosis in pathologic conditions is not controlled and can deteriorate organ function [7]. E.g. if apoptotic bodies are not eliminated, their membranes become permeable, leading to the release of cellular fragments into the extracellular space and triggering an inflammatory response. This process is called secondary necrosis [8]. In massive liver injury the ability of phagocytes to identify and clear apoptotic bodies is likely disturbed and an inflammatory response is observed despite an initial apoptotic stimulus in the liver [9, 10]. In addition, "diseased" hepatocytes can have adaptive alterations in their signal transduction pathways and organelle-regulated processes due to prolonged exposure to toxic stimuli such as excessive reactive oxygen species (ROS), toxic bile salts, free fatty acids and inflammatory cytokines. As a consequence they react differently than "normal" hepatocytes in response to these toxic stimuli (e.g., increased susceptibility of hepatocytes to primary necrosis instead of primary apoptosis, may lead to an exaggerated inflammatory response in the liver). Therefore, understanding the cellular processes and molecular signaling pathways regulating apoptosis and/or necrosis in (diseased) hepatocytes is essential to the development of new therapeutic strategies for (chronic) liver diseases.

Hepatocyte injury occurs upon exposure to toxic bile salts, inflammatory cytokines and increased levels of ROS. In this thesis, we have investigated the characteristic functions of steatotic and cholestatic hepatocytes using in vivo and in vitro models, respectively. Three models of hepatocyte cell death were studied: glycochenodeoxycholic acid (GCDCA)-induced cell death as a model for bile acid toxicity, tumor necrosis factor α (TNFα)/ actinomycin D (ActD, an inhibitor of transcription) as a model for cytokine-induced toxicity and menadione-induced cell death (menadione is a superoxide anion donor) as a model for oxidative stress-induced toxicity. These models are clinically relevant as these toxic stimuli are present at increased levels in most liver diseases.

Chapter 3 describes the susceptibility of the steatotic hepatocytes to apoptosis and necrosis. Hepatocyte necrosis is suggested to trigger an excessive inflammatory response in fatty livers, increasing the morbidity and mortality of NAFLD patients [11-14]. Previous studies in animal models of NAFLD suggested higher susceptibility of fatty heptocytes to endotoxin-induced injury and bile acid-induced necrosis [15, 16]. However, the underlying mechanisms of altered sensitivity of fatty hepatocytes are still a matter of debate. We used hepatocytes isolated from Zucker obese rats as a model of NAFLD [17]. Fatty hepatocytes showed clearly increased cellular necrosis shortly after the isolation, representing their vulnerability to necrosis in vivo as well. We hypothesized

that alterations in mitochondria-regulated processes play a role in this phenomenon in steatotic hepatocytes, as mitochondria play an important role in both apoptotic and necrotic cell death in hepatocytes [18-21]. An important step in the mitochondria-dependent cell death is mitochondrial permeability transition (mPT). Opening of the permeability transition pore (mPTP) in the inner membrane can trigger mPT. Opening of the mPTP is thought to initiate necrosis rather than apoptosis [22, 23]. Cyclophilin D, ANT and VDAC are suggested to be related to the mPTP [23-27] and it is reported that cells overexpressing cyclophilin D are hypersensitive to necrotic cell death [28]. In accordance, our findings indicate that cyclophilin D is up-regulated in fatty hepatocytes. Although ANTs are thought to be non-essential components of the mPTP, they do have an important role in regulating the permeability transition by modulating the sensitivity of the mPTP to Ca^{2+} and ANT ligands [27]. Cyclophilin D is thought to facilitate a calcium-triggered conformational change in the ANT converting it to an open pore [22]. Therefore, the up-regulation of Ant-1 in fatty hepatocytes can also play a role in the increased susceptibility of steatotic hepatocytes to necrosis. We also observed an increase in mRNA expression levels of Vdac-1 and Vdac-3 in steatotic hepatocytes but we were not able to detect a difference in protein expression of Vdac-1 and Vdac-3 between lean and fatty hepatocytes. Whether the increase in mRNA expression level of Vdac-1 or Vdac-3 has a role in the susceptibility of fatty hepatocytes to necrosis and/or apoptosis remains to be established. Fatty hepatocytes were clearly resistant to bile acid-induced apoptosis, whereas they showed equal sensitivity to TNFα/ActD-induced apoptosis. Previously, we have shown that GCDCA induces apoptosis in primary rat hepatocytes in a mitochondria-dependent manner while cytokine induces apoptosis in a death receptor-dependent manner [29, 30], suggesting that alterations in mitochondria-mediated pathways are crucial in regulating bile salt-induced cell death in hepatocytes. Therefore, the up-regulation of mPTP proteins may also play role in the resistance of fatty hepatocytes to bile acid-induced apoptosis and their susceptibility to bile acid-induced necrosis.

Steatotic hepatocytes are exposed to increased levels of free fatty acids and oxidative stress, thus it is very likely that they adapt themselves to the environment. Mitochondria, as one of the main organelles for lipid metabolism in hepatocytes, are exposed to these stimuli and therefore, alterations in mitochondria-regulated pathways are not unexpected. In accordance, our findings confirm the presence of such alterations in steatotic hepatocytes, thereby predisposing them to necrosis. The consequence of susceptibility to necrosis rather than apoptosis is that hepatocytes in fatty livers are more prone to necrosis-induced inflammation. Once inflammation occurs, the prognosis of NAFLD patients is worse [11, 13]. Our study suggests potential targets for therapy to

prevent necrosis in fatty livers (e.g. inhibition of the mPTP and in particular the mPTP regulator, cyclophilin D), and thereby protect fatty livers against inflammation and exacerbation of liver damage.

Chapter 4 describes the protective effects of angiotensin II (AT-II) against bile salt-induced apoptosis in primary rat hepatocytes. AT-II antagonists (ACEi and/or ARB) are considered as therapeutics for fibrotic liver diseases as they prevent HSC activation and proliferation [31]. However, the effect of AT-II antagonist therapy on "diseased" (e.g. cholestatic) hepatocytes has not been studied. Importantly, ACEi- and ARB-induced hepatocellular injury and liver damage have been reported previously [32-37]. Several mechanisms have been suggested for ARB-induced hepatotoxicity and cholestasis such as metabolic idiosyncrasy and immune mechanisms. However, the hypothesis that angiotensin II has hepatoprotective effects and ARBs sensitize hepatocytes to toxic stimuli has not been investigated. In our study, we used several apoptotic stimuli (bile salts, oxidative stress and cytokine) that are present in chronic liver diseases, to investigate the effect of AT-II specifically on diseased hepatocytes. Our data strongly demonstrate that AT-II has AT-1R-mediated hepatoprotective effects in hepatocytes which were exposed to cholestatic bile salts. These effects are prevented by several ARBs. Interestingly, AT-II protects hepatocytes against bile salt-induced apoptosis but not against TNFα- and menadione-induced apoptosis. This could be due to the induction of different signaling pathways in response to these apoptotic stimuli [29, 38-40]. We have provided evidence that the protective effects of AT-II are mediated via combined activation of protein kinase signal transduction pathways including ERK, PI3K, p38 MAPK and PKC. We have shown previously that taurine-conjugated UDCA (TUDCA)-induced activation of ERK, PI3K and p38 MAPK protects primary rat hepatocytes against bile salt-induced apoptosis [41]. Thus, it is likely that AT-II mediated activation of these protein kinases leads to the survival of cholestatic hepatocytes. In addition, we observed that AT-II reduces bile salt-induced CHOP expression. CHOP is highly induced during ER stress, activating the ER stress-induced apoptotic pathways [42]. CHOP deficiency reduces cholestasis-induced hepatocyte apoptosis [43]. Activation of the ERK1/2, PI3K/Akt and PKC pathways were found to attenuate ER stress-induced apoptosis and reduce CHOP expression, leading to cell survival [44-47]. Interestingly, inhibition of the ER-stress response by TUDCA is a hepatoprotective mechanism in liver disease [48]. Our data suggest that activation of these protein kinases in AT-II treated hepatocytes inhibits the ER stress response, leading to hepatocyte survival.

The hepatoprotective effect of AT-II against bile salt-induced apoptosis has great clinical relevance in AT-II antagonist therapy of patients with cholestatic liver fibrosis. Patients with chronic

cholestatic liver diseases (e.g., PSC, PBC) usually visit the clinicians when their disease is already advanced and the therapy has to start in already diseased livers containing cholestatic hepatocytes. Our data suggest that despite the beneficial effects of ARBs in the treatment of liver fibrosis, they may hypersensitize adjacent hepatocytes to several toxic bile salts such as taurolithocholic sulfate (TLCS) and GCDCA, leading to further loss of functional hepatocytes. Therefore, liver function should closely be observed during the AT-II antagonist therapy of cholestatic liver fibrosis, especially in the early stages of cholestatic liver disease. In addition, targeting AT-II antagonists specifically to hepatic stellate cells can be a good solution in order to protect functional hepatocytes during the treatment of liver fibrosis. Combined (taurine-conjugated) UDCA/ARBs therapy could serve as an alternative option to protect hepatocytes while treating liver fibrosis in cholestatic liver diseases, as UDCA has the same hepatoprotective effects as observed for AT-II in our study and UDCA is already used in clinic for patients with chronic cholestatic diseases.

In **chapter 5**, the involvement of Gαi-protein coupled receptors (GPCR) in regulating hepatocyte apoptosis is investigated. In the liver, lysophosphatidylcholine acts via a GαiPCR-dependent mechanism in regulating lipoapoptosis of hepatocytes [49]. Whether GαiPCR also plays a role in bile acid- or cytokine-induced apoptosis is not known. We report that pertussis toxin (PT), an inhibitor of Gαi-proteins, protects primary rat hepatocytes against apoptosis. The anti-apoptotic effect of PT is mediated via inhibition of GαiPCRs and is implicated in bile acid-induced apoptosis as well as in cytokine-induced apoptosis in primary rat hepatocytes. A crucial common step of both bile salt-induced apoptosis and cytokine-induced apoptosis in rat hepatocytes is ligand-independent transactivation of the epidermal growth factor receptor (EGFR, a tyrosine kinase receptor) [50-56]. Tyrosine kinase receptor transactivation has also been associated with GPCRs activation (reviewed in [57]). Given the anti-apoptotic effect of PT in rat hepatocytes in both models of apoptosis, we suggest that there is cross-talk between PT-sensitive GPCR/Gαi and the EGFR which leads to hepatocyte apoptosis. Indeed, it has been observed that PT pretreatment of hepatocytes inhibited taurodeoxycholic acid (TDCA)-induced activation of the EGFR [58]. It is also reported that PT-sensitive Gi proteins are uniquely involved in the signal transduction pathway mediating EGF-induced activation of phospholipase C-gamma (PLCγ) and Ca2+ mobilization (via tyrosine phosphorylation of the EGFR) in rat hepatocytes [59].Whether GCDCA-induced activation of the EGFR in primary hepatocytes is also PT-sensitive remains to be investigated.

Our data indicate that GPCRs/Gαi participate in several apoptotic signaling pathways in hepatocytes and that inhibiting the αi subunit of G-proteins is a very effective anti-apoptotic strategy in primary hepatocytes in vitro. Interestingly, we observed that PT has no protective effects

in bile salt- and cytokine-induced cell death in HepG2-rNtcp cells (hepatocellular carcinoma cell line).

The participation of GPCR/Gαi in hepatocyte apoptosis suggests new targets for drug innovation for (chronic) liver diseases and liver cancer treatment in future. E.g., activating death ligand-mediated apoptosis in cancer cells (e.g., TRAIL-, FasL-, TNF-mediated cell death) is suggested as an effective therapeutic strategy in the treatment of hepatocellular carcinoma [60]. However, this strategy may lead to the induction of apoptosis in adjacent hepatocytes and tissue damage. In this situation, an adjuvant anti-apoptotic therapy which will inhibit cell death in normal hepatocytes but has no effect on cancer cells may reduce the normal tissue damage. Our data suggest that GPCR/Gαi-based therapeutic strategies may serve as the anti-apoptotic adjuvant therapy, protecting normal tissue.

Chapter 6 describes a novel bile salt-induced signaling pathway mediating hepatocyte apoptosis including sphingosine kinase-1, sphingosine-1 phosphate (S1P) and S1P receptors (S1PR1 and S1PR2).

It is known that sphingosine kinase (SphK) isoenzymes as well as endogenous and exogenous S1P have opposing functions in different cells and different organs [61-65]. Liver cells are also not excluded from this enigma. S1P is proapoptotic via a receptor-independent pathway and anti-apoptotic via activation of S1P receptors in human hepatic myofibroblasts [66]. Sphk2 activation has been implicated in mitochondrial dysfunction and hepatocyte apoptosis after hepatic ischemia-reperfusion injury [67]. However, the effect of SphK1 and S1P in bile acid-induced apoptosis is currently unclear. In this study, we report that Ski II (a selective inhibitor of SphK1) protects primary rat hepatocytes specifically against GCDCA-induced apoptosis. We suggest that GCDCA-induced SphK1 activation and subsequently generated endogenous S1P mediates apoptosis mainly via S1P acting as a second messenger in hepatocytes and partly via $S1PR_1$-dependent signaling, whereas exogenous S1P protects hepatocytes against GCDCA-induced apoptosis via $S1PR_2$-dependent signaling. We demonstrate that the protective effect of Ski II is independent of the activation of specific kinases and of gene transcription, but Ski II inhibits GCDCA-induced $[Ca^{2+}]$ oscillations in rat hepatocytes. We also demonstrate that GCDCA-induced caspase-3 activity is dependent on the mobilization of intracellular $[Ca^{2+}]$ in rat hepatocytes.

In contrast to a previous report [68], we demonstrate that TNFα-induced apoptosis in rat hepatocytes is not SphK1 dependent. In this study [68], SphKs were inhibited by non-selective inhibitors (DMS and DHS), whereas we have used a selective inhibitor of SphK1 (Ski II). In addition, their experiments [68] were performed in human hepatoma cell lines, whereas we

used primary rat hepatocytes. The difference in our results could therefore be related to the use of different cell types and different inhibitors. Interestingly, we observed opposing functions for exogenous S1P versus endogenous S1P. Many reports indicate that S1P as a first messenger activates cell surface S1P receptors in a paracrine and/or autocrine manner (reviewed in [69]). We suggest that the main autocrine and/or paracrine role of S1P as a first messenger in our model is to activate $S1PR_2$-dependent signaling and induce cell survival. It has been shown that S1P via interaction with $S1PR_2$ induces phosphorylation of ERK1/2 and Akt in primary rat hepatocytes [70]. We have previously shown that activation of these pathways contributes to hepatocyte survival [41]. Therefore, these mechanisms could lend a clue to explain the protective effect of exogenous S1P against GCDCA-induced apoptosis. The activation of $S1PR_1$-dependent signaling by autocrine S1P during GCDCA-exposure is then pro-apoptotic for rat hepatocytes. However, this pathway does not seem to contribute importantly to hepatocyte apoptosis as the antagonist of $S1PR_1$ reduces GCDCA-induced apoptosis only by 20%. Therefore, the evidence that GCDCA-induced apoptosis is a SphK1-dependent event suggests that the endogenously generated S1P acts as a second messenger to promote cell apoptosis in rat hepatocytes. S1P is suggested to act as a second messenger to mobilize calcium from the ER [71, 72]. GCDCA-induced apoptosis in rat hepatocytes has been shown to be accompanied by increased intracellular $[Ca^{2+}]$ levels, likely from ER stores [73]. In our experiments, the GCDCA-induced increase in $[Ca^{2+}]$ in rat hepatocytes is observed as $[Ca^{2+}]$ oscillations. Interestingly, we show that GCDCA induces $[Ca^{2+}]$ oscillations in a SphK1-dependent manner which is not mimicked by exogenous S1P. In addition, we show that GCDCA-induced apoptosis is dependent on intracellular calcium mobilization, since GCDCA-induced apoptosis was reduced in hepatocytes loaded with BAPTA (a chelator of intracellur $[Ca^{2+}]$). Therefore, we suggest that GCDCA-induced endogenous S1P can act as a second messenger to induce $[Ca^{2+}]$ oscillations in rat hepatocytes. Inhibition of the SphK1 activation inhibits GCDCA-induced apoptosis as well as GCDCA-induced $[Ca^{2+}]$ oscillations. In summary, our data suggest that GCDCA-induced SphK1 activation is among the initial events in the bile acid-induced apoptotic cascade. Therefore, inhibiting SphK1 in hepatocytes could be a very effective anti-apoptotic strategy in cholestatic liver diseases.

Conclusions and future perspectives

Knowledge about the cellular mechanisms regulating death and survival of liver cells is of clinical and scientific interest for developing new therapeutic strategies. This thesis describes that hepatocyte

death and survival is regulated at the cross-roads of intracellular organelles (e.g., mitochondria and endoplasmic reticulum), membrane-bound receptors (such as GPCRs and EGFR) and cell survival signaling pathways (such as ERK, PI3K and PKC) (Figure1).

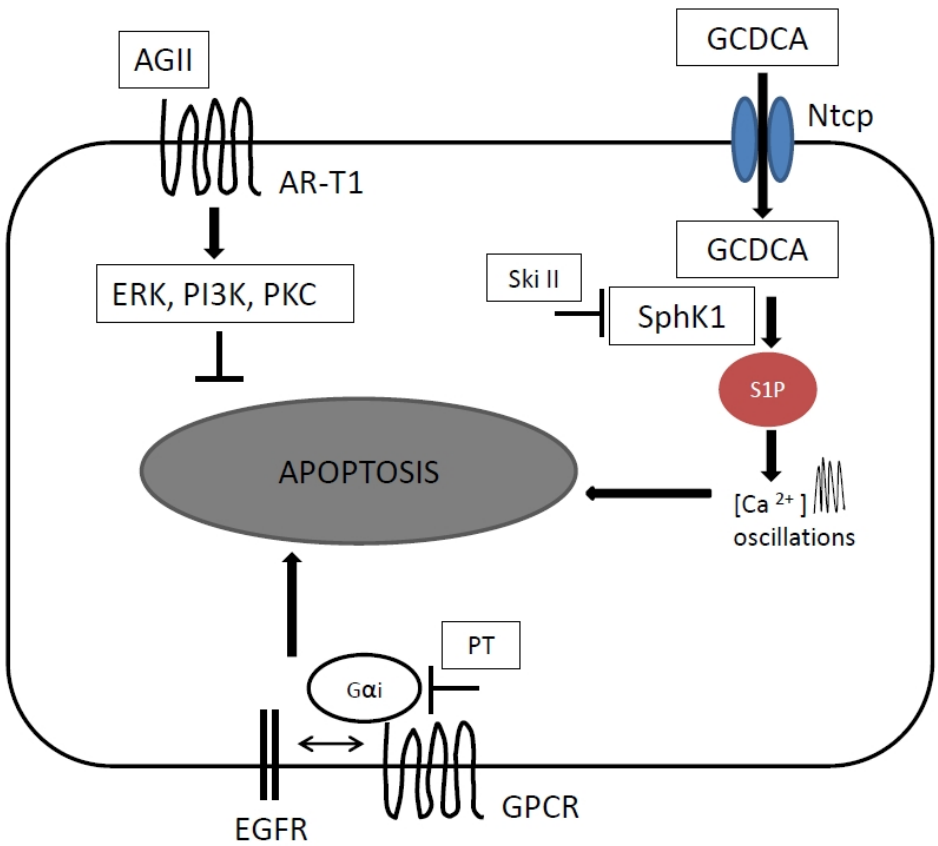

Figure1: Schematic picture summerizing the findings of this thesis. Hepatocyte death and survival is regulated at the cross-roads of intracellular organelles, membrane-bound receptors (such as GPCRs and EGFR) and cell survival signaling pathways (such as ERK, PI3K and PKC).

GαiPCRs are generally transducing apoptotic signals in hepatocytes. However, S1PR2 receptor (also a GαiPCR) can also transduce cell survival signals upon activation by extracellular ligands such as S1P. Small lipid molecules such as S1P act as signaling molecules in hepatocytes, thereby playing dual roles in determining the fate of hepatocytes (depending on their localization in hepatocytes). Cross-talk between GPCRs and tyrosine kinase receptors such as the EGFR is also of great interest, as this pathway appears to be a common mechanism for several toxic stimuli in liver diseases (such as bile salts and cytokines). Thus interference with this step may be a beneficial anti-apoptotic strategy in chronic liver diseases (such as cholestasis) with an inflammatory component. The fact that hepatocellular carcinoma cells may not share all the signaling components of apoptosis with primary hepatocytes suggests new targets for adjuvant anti-apoptotic therapies in tumor treatment to reduce normal tissue damage during anti-tumor therapy. Diseased hepatocytes, however, are

likely to have altered characteristics and /or responses when exposed to toxic stimuli. This has important consequences for drug innovation, since an anti-apoptotic strategy may not be as efficient in reducing liver inflammation as an anti-necrotic therapy in e.g. steatotic liver disease. Finally, targeting therapies to diseased cells (e.g. activated stellate cells and myofibroblasts in liver fibrosis) may show better outcome in chronic liver diseases and reduce toxic side-effects. E.g. ARBs, considered as therapy for liver fibrosis, may have adverse effects in adjacent functional hepatocytes, thereby leading to further loss of liver function.

Although we have used 'diseased' hepatocytes in some experiments (e.g. fatty hepatocytes), we did not use 'real' cholestatic hepatocytes. Cholestatic conditions were mimicked by exposing normal hepatocytes to cholestatic bile salts. Future studies should focus more on the use of 'in vivo diseased' hepatocytes to validate the findings obtained in this thesis. After all, when patients with liver disease visit clinicians their liver cells are not normal anymore but are 'diseased'. Moreover, as all pathways and organelles are somehow interconnected and interact with each other in a reciprocal fashion future studies should also investigate the consequences of interrupting a single apoptotic pathway on another pathway, e.g. mitochondria-targeted therapy on ER-stress related phenomena in (e.g., steatotic) hepatocytes.

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Chapter 8

Appendices

Nederlandse Samenvatting

Written in English by Golnar Karimian

Translated by Manon Buist-Homan

Cholestatische en steatotische leverziekten hebben een significant aandeel in het totaal van leverziekten. Zowel cholestatische (zoals primaire biliaire cirrose: PBS, en primaire scleroserende cholangitis: PSC) en steatotische (zoals niet alcoholische steatohepatitis: NASH) leverziekten zijn geassocieerd met hoge morbiditeit en sterftecijfers. Bestaande therapieën zijn of niet erg effectief (bijv. steroïden of UDCA in het geval van PBC) of zijn geassocieerd met hevige moeilijkheden (bijv. levertransplantatie: orgaan donor te kort, orgaan afstoting, etc.).

Eén van de redenen voor het te kort aan effectieve therapie voor cholestatische en steatotische leverziekten is het ontbreken van gedetailleerde kennis van de pathogenetische mechanismen van deze ziekten. Beide ziektegroepen worden gekarakteriseerd door een geleidelijk en progressief verlies van gezonde hepatocyten wat leidt tot leverontsteking, fibrogene en eindstadium leverziekte. Verlies van cellen kan via apoptose of necrose (of een tussenvorm) gaan. Echter bestaat er een uitgebreide controverse tussen de basis kennis als de dominante vorm van celdood in deze ziekten. Echter, elke interventie of therapeutische strategie zou effectief moeten zijn in 'zieke' (d.w.z. steatotische of cholestatische) hepatocyten, want patiënten met chronische leverziekten komen vaak pas bij de dokter wanneer hun ziekte al vergevorderd is. Het doel van dit proefschrift is de mechanismen van celdood in (zieke) hepatocyten op te helderen en op die manier strategieën te ontwikkelen om de (zieke) hepatocyten te beschermen en leverschade te voorkomen.

In **hoofdstuk 2** wordt de actuele kennis van celdood in hepatocyten met speciale nadruk op intracellulaire organel gemedieerde celdood en membraan gebonden receptoren die apoptose reguleren beoordeelt.

Hoofdstuk 3 beschrijft de gevoeligheid van de steatotische hepatocyten op apoptose en necrose. Er wordt voorgesteld dat necrose van hepatocyten leidt tot een excessief inflammatoire respons in vette levers, verhoogde morbiditeit en sterftecijfers van NAFLD patiënten. Echter de onderliggende mechanismen van veranderde gevoeligheid van vette hepatocyten zijn nog steeds onderwerp van discussie. Hepatocyten geïsoleerd van dikke Zucker ratten zijn gebruikt als model voor NAFLD. Vette hepatocyten laten duidelijk verhoogde cellulaire necrose zien kort na de isolatie, wat representatief is voor hun gevoeligheid voor necrose in vivo. Onze hypothese is dat veranderingen in mitochondrieel gereguleerde processen een rol spelen in dit fenomeen in steatotische hepatocyten want mitochondria spelen een belangrijke rol in zowel apoptotische als necrotische celdood. Een belangrijke stap in de mitochondrieel afhankelijke celdood is de mitochondrial permeability transition pore (mPT). Het openen van de mitochondrial permeability transition pore (mPTP) in de binnenste membraan kan leiden tot mPT. Van het openen van de mPTP wordt gedacht dat het leidt tot necrose i.p.v. apoptose. Van Cyclophilin D, ANT en VDAC wordt gedacht dat ze

gerelateerd zijn aan de mPTP. Wij zagen een verhoging op mRNA niveau van Vdac-1 en Vdac-3 in steatotische hepatocyten. Vette hepatocyten zijn duidelijk resistent tegen galzout geïnduceerde apoptose, maar tonen een gelijke gevoeligheid voor TNFα/ActD geïnduceerde apoptose. Onze therapie laat potentiele doelen zien voor therapie om necrose te voorkomen in vette levers (bijv. remmen van de mPTP en met name de mPTP regulator, cyclophilin D) en daarmee vette levers te beschermen tegen ontsteking en een toename van de leverschade.

Hoofdstuk 4 beschrijft het beschermende effect van angiotensine II (AT-II) tegen galzout geïnduceerde apoptose in primaire rat hepatocyten. AT-II antagonisten (ACEi en/of ARB) worden gezien als medicijnen voor fibrotische leverziekten want zij houden de activatie en proliferatie van HSC tegen. Hoewel het effect van AT-II antagonist therapie op 'zieke'(cholestatische) hepatocyten nog niet bestudeerd is. In deze studie hebben we verschillende aopototische stimuli (galzouten, oxidatieve stress en cytokine) gebruikt die aanwezig zijn in chronische leverziekten om het effect van AT-II specifiek op zieke hepatocyten te onderzoeken. Onze data laat duidelijk zien dat AT-II een AT-1R gemedieerd beschermend effect heeft op hepatocyten die blootgesteld zijn aan cholestatische galzouten. Deze effecten worden voorkomen door verschillende ARBs. AT-II beschermt hepatocyten tegen galzout geïnduceerde apoptose maar niet tegen TNFα- en menadion geïnduceerde apoptose. Wij laten zien dat de beschermende affecten van AT-II gemedieerd zijn via gecombineerde activatie van proteïne kinase signaal transductie routes waaronder ERK, PI3K, p38 MAPK en PKC. Daarnaast zien we dat AT-II de galzout geïnduceerde CHOP expressie reduceert. CHOP is erg geïnduceerd tijdens ER stress, wat leidt tot activatie van de ER-stress geïnduceerde apoptotische routes. Een CHOP tekort reduceert cholestase geïnduceerde apoptose van de hepatocyten. Activatie van ERK1/2, PI3K/Akt en PKC routes blijkt de ER stress geïnduceerde apoptose te verzwakken en de CHOP expressie te reduceren wat leidt tot cel overleving. Onze data laat zien dat activatie van deze proteïne kinases in AT-II behandelde hepatocyten de ER stress response remmen wat leidt tot hepatocyt overleving. Het lever beschermende effect van AT-II tegen galzout geïnduceerde apoptose is van groot klinisch belang in AT-II antagonist therapie bij patiënten met cholestatische leverfibrose. Patiënten met chronische cholestatische leverziekten (bijv. PSC, PBC) bezoeken hun dokter normaal gesproken pas wanneer hun ziekte al vergevorderd is en de therapie moet dan starten in een lever die al ziek is en cholestatische hepatocyten bevat. Onze data laat zien dat ondanks de voordelige effecten van ARBs in de behandeling van leverfibrose ze de aangrenzende hepatocyten overgevoelig maken voor verscheidene toxische galzouten zoals taurolithochol sulfaat (TLCS) en GCDCA, wat leidt tot het verder verlies van functionele hepatocyten. Daarom moet de leverfunctie goed in de gaten worden gehouden tijdens de AT-II antagonist therapie van de cholestatische

leverfibrose, met name tijdens de eerste stadia van de cholestatische leverziekte. Daarnaast kan het richten van AT-II antogonisten specifiek tot hepatische stellaat cellen een goede oplossing zijn om functionele hepatocyten tijdens behandeling van leverfibrose te beschermen. Gecombineerde (taurine geconjugeerde) UDCA/ARBs therapie zou een goed alternatief kunnen zijn om hepatocyten te beschermen tijdens de behandeling van leverfibrose in cholestatische leverziekten want UDCA heeft hetzelfde lever beschermende effect als we zien bij AT-II in dit proefschrift en UDCA wordt al in de kliniek gebruikt bij patiënten met chronische cholestatische ziekten.

Hoofdstuk 5 beschrijft het beschermende effect van pertussis toxine (PT), de Gαi-proteïne remmer, tegen galzout en cytokine geïnduceerde apoptotische celdood. Een cruciale stap in zowel galzout geïnduceerde apoptose en cytokine geïnduceerde apoptose in rat hepatocyten is de ligand afhankelijke transactivatie van de epidermale groeifactor receptor (EGFR, een tyrosine kinase receptor). Vanwege het anti- apoptotische effect van PT in rat hepatocyten in beide modellen van apoptose denken wij dat er een interactie is tussen PT gevoelige GPCR/Gαi en de EGFR wat leidt tot apoptose in de hepatocyt. Onze data laat zien dat GPCRs/Gαi deelnemen in verschillende apoptotische signaal routes in hepatocyten en dat het remmen van de αi subeenheid van G-proteïne een zeer effectieve anti-apoptotische strategie is in primaire hepatocyten in vitro. We hebben gezien dat PT geen beschermende effecten in galzout en cytokine geïnduceerde celdood in HepG2.rNtcp cellen (hepatocellulaire carcinoma cellijn) heeft.

Het deelnemen van GPCR/Gαi in apoptose van hepatocyten geeft nieuwe ideeën voor medicijn ontwikkeling voor (chronische) leverziekten en lever kanker behandeling in de toekomst. Bijvoorbeeld de activering van dood ligand gemedieerde apoptose in kanker cellen (bijv. TRAIL, FasL, TNF gemedieerde celdood) kan een effectieve therapeutische strategie zijn in de behandeling van hepatocellulaire carcinoma. Hoewel deze strategie kan leiden tot de inductie van apoptose in aangrenzende hepatocyten en weefsel schade. In deze situatie, een aanvullende anti-apoptotische therapie welke celdood in normale hepatocyten remt maar geen effect heeft op kanker cellen zal de schade in normaal weefsel reduceren. Onze data laat zien dat de op GPCR/Gαi gebaseerde therapeutische strategieën kan dienen als een anti-apoptotisch aanvullende therapie, om normaal weefsel te beschermen.

Het begrijpen van galzout geïnduceerde signaal routes is belangrijk voor de ontwikkeling van nieuwe medicijnen voor cholestatische afwijkingen. **Hoofdstuk 6** laat een nieuwe galzout geïnduceerde signaal route zien, die bestaat uit sphingosine kinase-1 (SphK1), sphingosine-1 fosfaat (S1P, een intracellulair lipide molecuul die zich gedraagt als second messenger) en S1P receptoren in primaire hepatocyten. Het is bekend dat sphingosine kinase (SphK) iso-enzymen

maar ook endogeen en exogeen S1P tegengestelde functies in verschillende cellen en verschillende organen hebben. SphK2 activatie is betrokken bij mitochondreel disfunctioneren en apoptose van hepatocyten na hepatische ischemie reperfusie schade. Hoewel het effect van SphK1 en S1P in galzout geïnduceerde apoptose momenteel onduidelijk is. In deze studie laten we zien dat Ski-II (een selectieve remmer van SphK1) specifiek primaire rat hepatocyten beschermd tegen GCDCA geïnduceerde apoptose. Wij zeggen dat GCDCA geïnduceerde SphK1 activatie en de daarop volgend de endogene generatie van S1P gereguleerde apoptose voornamelijk via S1P als second messenger gaat in hepatocyten en gedeeltelijk via S1PR1 afhankelijke signalering, waar exogeen S1P hepatocyten beschermd tegen GCDCA geïnduceerd apoptose via S1PR2- afhankelijke signalering. Wij laten zien dat het beschermende effect van Ski-II onafhankelijk is van de activatie van specifieke kinases en van gen transcriptie maar Ski-II remt GCDCA geïnduceerde [CA2+] fluctuaties in rat hepatocyten. We laten ook zien dat GCDCA geïnduceerde caspase-3 activiteit afhankelijk is van mobilisatie van intracellulair [Ca2+] in rat hepatocyten. Samenvattend laat onze data zien dat GCDCA geïnduceerde SphK1 activatie één van de belangrijkste onderdelen is van de galzout geïnduceerde apoptotische cascade. Daarom kan het remmen van SphK1 in hepatocyten zeer effectief zijn als anti-apoptotische strategie in cholestatische leverziekten.

Conclusies en toekomst perspectieven

Kennis van de cellulaire mechanismen die het afsterven en overleving van levercellen reguleert is van klinisch en wetenschappelijk belang voor de ontwikkeling van nieuwe therapeutische strategieën. Dit proefschrift beschrijft dat hepatocyten celdood en overleving zijn gereguleerd door middel van intracellulaire organellen (zoals mitochondria en het endoplasmatisch reticulum), membraan gebonden receptoren (zoals GPCRs en EGFR) en cel overlevingsroutes (zoals ERK, PI3K en PKC). Het feit dat hepatocellulaire carcinoma cellen niet dezelfde signalerings onderdelen van apoptose delen met primaire hepatocyten laat nieuwe doelen zien voor aanvullende anti-apoptotische therapieën in tumor behandeling om schade aan normaal weefsel te voorkomen tijdens anti tumor therapie. Zieke hepatocyten daarentegen hebben veranderde karakteristieken en/of reacties wanneer ze aan toxische stimuli blootstaan. Dit heeft belangrijke consequenties voor medicijn ontwikkeling omdat een anti-apoptotische strategie niet zo efficiënt is in gereduceerde leverontsteking als een anti-necrotische therapie in bijvoorbeeld steatotische leverziekten. Tot slot, therapieën gericht tegen zieke cellen (bijv. geactiveerde stellaat cellen en myofibroblasten in leverfibrose) hebben een betere uitkomst in chronische leverziekten en reduceren toxische bijwerkingen. Bijvoorbeeld ARBs,

gezien als therapie voor leverfibrose, kunnen nadelige effecten hebben in aangrenzende functionele hepatocyten wat leidt tot een verder verlies van de lever functie.

Ondanks we 'zieke' hepatocyten hebben gebruikt in sommige experimenten (bijv. vetten hepatocyten) hebben we geen gebruik gemaakt van echte cholestatische hepatocyten. Cholestatische condities werden nagebootst door het blootstellen van normale hepatocyten aan cholestatische galzouten. Toekomstige studies zouden de nadruk meer moeten leggen op het gebruik van in vivo ziek gemaakte hepatocyten om de bevindingen van dit proefschrift te controleren. Daarnaast als alle routes en organellen met elkaar verbonden zijn en met elkaar communiceren in een wederzijdse manier dan moet toekomstig onderzoek ook de gevolgen van het onderbreken van één enkele apoptotische route op een andere route onderzoeken, bijvoorbeeld mitochondria gerichte therapie op ER-stress gerelateerd fenomeen in (bijv. steatotische) hepatocyten.

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Chapter 8

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Biography

Golnar Karimian was born in Shiraz, Iran, in 1981. At the age of 11, she attended the school for highly talented students where she studied natural sciences. In 1999, at the age of 18, she was accepted in Tehran University of Medical Sciences. She therefore moved to Tehran to study medicine. Golnar graduated from Medical faculty in October 2007. Shortly afterwards in December 2007, she went to the University of Groningen in order to persue her carrier in science and research. She worked as PhD student at the department of Hepatology and Gastroeneterology under supervision of Prof. Han Moshage and Prof. Klaas Nico Faber. Her research was conducted on hepatocyte cell death in chronic and acute liver diseases. She finished her PhD in January 2012. Currently, she is following her research interest in the field of liver regeneration as post-doctoral researcher at the department of Surgery, section liver transplantation and hepatobiliary surgery within the surgical reasearch laboratory in University Medical Center Groningen. In her spare time, Golnar likes to read, to watch movies and to paint.